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PLUMAGE SEQUENCE IN A CASE OF HEMILATERAL ASYMMETRY IN MALE HYBRID FOWL[1]

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THE exhibition of lateral asymmetry by an individual becomes of special interest when it is manifest in a system subject to continued regeneration; for, if first encountered at an early age, it may be seen that, if the asymmetry is static, identical pattern succeeds identical pattern, whether or not it fluctuates in degree with each changing developmental level of the organism or even if it may perhaps become completely obliterated in the later life of the originally asymmetric individual.

The observation in this laboratory of a young cockerel showing a distinct hemilateral asymmetry in the distribution of the pigment patterns of its feathering suggested promise for study in such respects, since the plumage, as is well known, represents something of an embryonic system, persisting during life and giving rise to series of successive feather generations. The appearance of the first juvenile plumage and that of its next successor stage in this bird have been briefly described in an abstract (Juhn, 1942); here the material will be dealt with more fully and with special reference to the patterns developed in the successive feathers proliferated by follicles of strictly comparable opposite position in the body.

DESCRIPTION

The cockerel was brought into the laboratory from the range on June 10, 1941, at which time it was about 8–10 weeks old and still in the juvenile feathering. It had lost its identifying bands, so that nothing absolute can be said of its parentage; but the appearance of its plumage and the known composition of some of the University of Maryland flock matings suggest a hybrid from a Barred Plymouth Rock male and New Hampshire female cross.

The juvenile plumage was barred black and white, with red patches, which were much larger on the right side of the body. In certain regions, notably in the head, back, and saddle, the mid-line of the body separated the barred plumage of the left from the red portions on the right.

The cockerel showed only a very slight difference in size between the body halves, and there was no apparent varia-

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tion in the color of the irises. The feet and shanks were cream colored, with perhaps a slightly more reddish tint on the right.

Records were made of the juvenile plumage in all regions; the bird was in full molt; hence no pluckings for specific regenerations were made, except the right and left secondaries No. 11 and samples from the right and left margin of the saddle.

The bird, which had been kept in an individual cage for observation, became ill toward the middle of August and, upon development of paralysis, was sacrificed on August 28, 1941. At autopsy, records were made of the superficial condition of the internal organs, and then the testes, adrenals, hypophysis, thyroid, sciatics, and irises were fixed in Bouin's solution. The entire skeleton save the head was placed in formalin for later measurement. The wings were removed from the body and extended in order to facilitate study of the individual feathers. The skins of the dorsal and ventral surfaces with attached feathers were spread and dried separately.

TABLE 1

COMPARISON OF SKELETAL LENGTHS IN MILLIMETERS IN THE RIGHT AND LEFT APPENDAGES OF THE HYBRID COCKEREL, M-917

Appendage	Right	Left
Thigh	109.0	109.5
Tibia	157.0	163.0
Tarsus	122.0	128.5
Middle toe	74.0	74.0
Hind toe	22.0	23.0
Upper arm	93.5	95.0
Forearm	98.0	100.0
Hand	67.0	67.0

None of the internal organs gave evidence of asymmetry save the testes, where the variation was noticeable: the left gland measured 189 mm. in length and 86 mm. in width; on the right the corresponding dimensions were 137 and 69 mm. Sections through representative levels of either testis showed only immature tubules; the spermatogonia seemed abnormal in some regions, although this is to be attributed to the pathological condition of the cockerel at autopsy.

The skeletal lengths of the appendages were taken with a pair of large calipers in millimeters; these measurements are entered in Table 1. The very slight difference in favor of the left side occurs in the tibia, the tarsus, and the forearm. The localization of the size difference in these skeletal components may be an indication of the embryonic time of onset of the differential between right and left.

For the plumage in general, the pigment pattern recorded for the juvenile feathers of the various regions is compared with the pattern of feathers which replaced those molted or plucked in the corresponding regions. In the wings, comparisons were possible between juvenile and adult feathers from given individual follicles of identical position on the right and left; these records will be given in more detail farther along, the other regions being described as follows:

Hock.—Juvenile feathers were barred, with some red, the red segments being more extensive on the right. Adult feathers on the left were almost all barred; on the right there were many red feathers, most often having a barred apex and a solid red base.

Thigh.—Juvenile feathers were barred, with traces of red, the red being more obvious on the right. Adult feathers on the left were practically all barred, with very few feathers having red fluff and a red rhachis stripe extending about halfway up the feather; on the right there was much red, usually forming the basal portion of the feather but sometimes continuing apically along the rhachis.

Saddle.—Juvenile feathers were barred, with some red segments to the right of the mid-line. Adult feathers replacing the juveniles plucked on June 10, 1941, and those following in natural molt were all strictly male—long, acuminate, and with associated with the decrease in barbulation of the apical barb segments. On the right side the majority of the feathers were red, frequently with a barred black-and-white tip, through which red barbs sometimes extended to the feather apex.

TABLE 2

COMPARISON OF THE PATTERN DISTRIBUTION AT DATE OF AUTOPSY (8-28-41) IN THE RIGHT AND LEFT PRIMARIES AND CORRESPONDING MAJOR COVERTS AND UNDERCOVERTS OF THE HYBRID COCKEREL, M-917

	No. of Feather																			
	Right 1*									10	Left 1									10
Primaries	b	b	b	b	-†	R‡	R	R	R	R	b	b	b	b	B	B	B	R	B	B
Major coverts	b	R	R	R	R	R	R	R	b	b	b	b	b	B	B	B	B	R	b	b
Undercoverts	-	-	b	b	B	B	B	B	R	-	b	b	B	B	B	B	B	R	B	B

* The feathers (1–10) are counted from the direction of the wing tip toward the body.

† - = Empty follicle.

‡ Capitals indicate feathers of the present generation. Small letters identify feathers of the preceding generation which had not been molted. *B* and *b*, barred or partly barred and black; *R* and *r*, red or partly red, and barred.

TABLE 3

COMPARISON OF THE PATTERN DISTRIBUTION AT DATE OF AUTOPSY (8-28-41) IN THE RIGHT AND LEFT SECONDARIES AND CORRESPONDING MAJOR COVERTS AND UNDERCOVERTS OF THE HYBRID COCKEREL, M-917

	No. of Feather																					
	Right 11*										22	Left 11										22
Secondaries	R†	r	R	R	R	R	R	R	-‡	-	b	r	b	b	r	B	B	B	B	B	B	B	r	B
Major coverts	-	R	R	R	R	R	R	R	R	R	R	R	B	B	R	R	R	R	B	B	B	B	B	B
Undercoverts	-	B	B	B	B	B	R	R	R	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B

* The feathers (11–22) are counted from the direction of the wing tip toward the body.

† Capitals indicate feathers of the present generation. Small letters identify feathers of the preceding generation which had not been molted. *B* and *b*, barred or partly barred and black; *R* and *r*, red or partly red and barred.

‡ - = Empty follicle.

a lacy margin. On the left side, the feathers were barred black and white with narrow barring, or the feather tips alone were barred black and white, with the basal segment composed of a greenish-black rhachis stripe, with cream, yellow, or orange margin. The orange of these adult feathers was much more brilliant than the corresponding rusty red of the juvenile; this development appears

Breast.—Juvenile feathers were barred, with some red segments in both tracts; these segments were more extensive on the right side. Adult feathers on the left side were, for the most part, barred; but occasional red feathers were present. In some cases the barring did not reach the feather edge, the margin being white. On the right side, red feathers or feathers with red basal segments were much more

frequent than wholly black-and-white barred ones. Also, even where the feather tip was barred, a little red almost invariably extended to the apex as a continuation of a red rhachis or as red barbs proceeding from a red shaft, which, at this point, was itself turning black or white, and, in rare instances, the distal segment of an individual black or white barb was red. On close scrutiny this condition was not found on the left side. The white feather margin noted on the left side was of more frequent occurrence on the right side, and the barb portion involved was more extensive.

Wings.—For the primaries, secondaries, and corresponding major coverts and undercoverts, records were obtained of the individual feathers at the time of the first observation and again at date of autopsy. The data from these records are arranged in tabular form (Tables 2–6) and give a strict comparison between the right and left sides. First-generation feathers are indicated by small letters, second-generation feathers by capitals. "Red" is taken to include all feathers showing red segments, and "barred" includes feathers which may also have black regions.

TABLE 4

COMPARISON OF THE PATTERN SUCCESSION IN INDIVIDUAL FOLLICLES OF THE RIGHT AND LEFT PRIMARIES, SECONDARIES, AND TERTIARIES OF THE HYBRID COCKEREL, M-917

		No. of feather																						
		1*																						25
Right	6-10-41.....	b†	b	b	b	b	b	b	b	b	b	r	r	b	b	b	b	b	b	b	r	b	r	r	b	r
	8-28-41.....	b	b	b	b	-‡	R	R	R	R	R	R	r	R	R	R	R	R	R	-	-	b	r	r	b	r
Left	6-10-41.....	b	b	b	b	b	b	b	b	b	b	b	r	b	b	b	b	b	b	b	r	b	b	b	r	b
	8-28-41.....	b	b	b	b	B	B	B	R	B	B	B	r	B	B	B	B	B	B	B	r	b	b	b	r	b

* The feathers (1–25) are counted from the wing tip toward the body. The upper date indicates the first observation; the lower date the last record, obtained at autopsy.

† Feathers of the first generation (*small letters*) are entered in the upper line for right and left. Feathers of the second generation (*capitals*) are entered in the lower line. Where a feather was not molted, it appears again in the lower line. *B* and *b*, barred or partly barred and black; *R* and *r*, red or partly red and barred.

‡ - = Empty follicle.

TABLE 5

COMPARISON OF FEATHER LENGTHS IN MILLIMETERS IN THE RIGHT AND LEFT PRIMARIES AND COVERTS OF THE HYBRID COCKEREL, M-917, AT DATE OF AUTOPSY

		No. of Feather									
		1									10
Primaries	right.....	132	145	153	162	27*	119y	140	152	156	151
	left.....	140	150	159	166	36y†	106y	145	162	168	159
Coverts	right.....	42	66	83	81	82	83	85	81	62	57
	left.....	42	67	62	82	83	83	87	88	75	60

* Injured young feather.

† y = Young feather.

Table 2 illustrates the pattern distribution at date of autopsy between the right and the left primaries and their coverts. The preponderance of red on the right and of barred on the left is clearly apparent.

Table 3 shows this same situation for the secondaries and their coverts; the greater frequency of red on the right, as against red on the left, is equally distinct.

In Table 4, feathers of the first and second generation are entered for the individual follicles of the right and left primaries, secondaries, and coverts. This table demonstrates two distinct points: (1) the initial difference between the right and left sides, with the right showing somewhat more red, and (2) the replacement of barred first-generation by red second-generation feathers.

The tendency toward the replacement of barred black-and-white by red feathers as the development of the cockerel proceeded was a feature not only of the wings but of all regions of the body. It was more pronounced on the right side, which markedly increased the degree of initial asymmetry of the plumage.

In order to determine other possible differences between the feathers of the right and the left wings, the feather lengths at date of autopsy were taken. The larger feathers were measured in the sockets and the smaller ones upon removal.

Table 5 gives the feather lengths of the primaries and their coverts. On both sides, primaries 1–4 belong to the first, primaries 5–10 to the succeeding generation. In all the fully formed feathers, the ones of the left side are longer. However, the left one is the shorter of the pair where two corresponding feathers of each side are in growth (No. 6).

TABLE 6

COMPARISON OF FEATHER LENGTHS IN MILLIMETERS IN THE RIGHT AND LEFT SECONDARIES AND COVERTS OF THE HYBRID COCKEREL, M-917, AT DATE OF AUTOPSY

		No. of Feather											
		11											22
Secondaries	right....	103	139	141	139	132y*	118	91y	65y	34y	-†	134	123
	left.....	-	146	145	139y	131y	94y	74y	51y	-	145	143	128
Coverts	right....	93	99	107	104	106	105	107	103	99	96	94	91y
	left.....	-	99	112	110	111	110	110	104	103	103	95	83y

*y = Young feather.

†- = Empty follicle.

Of the coverts, on the right Nos. 1, 9, and 10 are of the first generation; on the left, Nos. 1, 2, 3, 9, and 10. Feathers 9 and 10 show a greater length on the left, but there is no difference between the others. In Table 6 the lengths of the secondaries and their coverts are entered. On the right, secondaries Nos. 12, 21, and 22 belong to the first generation and on the left, secondaries Nos. 12, 20, 21, and 22. Among the fully formed feathers, the left ones are again somewhat longer; but, of pairs still in growth, the left ones are shorter than those of the right of the corresponding position. The coverts are all of the second generation. In general, the feathers of the left are very slightly longer; but, of the pair in growth (No.

22), the left partner is again the shorter feather.

Tables 5 and 6 (see also Tables 2, 3, and 4) show that in the left wing there is a greater number of persistent first-generation feathers. This is a clear indication that molting commenced at a later date on the left than on the right.

Barb lengths were measured at 1-cm. intervals from the apex to the base of selected feathers of corresponding position in the right and the left wing, primaries 8 and 9, secondaries 15, and major coverts 12, 16, and 22. No differences in barb length were found when the corresponding vane-halves of opposite feathers were compared; hence the tables of these measurements are not included.

DISCUSSION

The description given in the preceding pages of the two successive plumages of the hybrid cockerel and the illustration in Plate I of its appearance as a juvenile both show clearly that all pigment pattern elements were represented from the first in all regions of the body of this male fowl and that the hemilateral asymmetry of its feathering derived from an unequal distribution of these elements to the right and left of the mid-line.

Since an all-over identity of the genes for the distinct feather pigments may, accordingly, be assumed here, the inquiry into the origins and the evolution of the asymmetry in this case will be guided from a more properly physiological point of view; however, for an account of other instances of asymmetries in birds, explained in terms of alterations within the genotype, reference is made to a recent review by Hollander (1944).

A physiological explanation of a special case of asymmetry, of hemilateral gynandromorphism, postulating subfunctional ovarian activity associated with differences in the rates of metabolic processes in the two sides of the body, was advanced by Lillie (1931). Lillie's interpretation was founded upon the experimental results of Juhn and Gustavson (1930), who found that, by controlling the amounts of injection, estrogen-induced effects could be restricted to feather areas of slower axial growth, as well as upon the later demonstration by Lillie and Juhn (published in full in 1932) that similar principles applied in the individual feather.

In Lillie's hypothesis the differentiating role of relative estrogen concentrations is, of course, recognized, but it is equally clearly stated that fundamental to any possible separation of modification are the initial differences between the parts affected. This second clause extends the application of the theory to far wider fields than asymmetries mediated through estrogen participation, as also recognized by Lillie in his discussion of Crew's case of hemilateral asymmetry in male fowl (Lillie, 1931, p. 390).

In the purely cock-feathered bird discussed here, fluctuating sex hormone levels are not involved in the asymmetry shown in the feathering, which may best be interpreted in comparison with observations on the relation of changing rates of axial feather growth to pattern development in other male hybrids and male purebred particolored fowl.

In the Brown Leghorn, a breed homozygous for feather pigmentation, feathers of the main tail show a rapid increase in their axial rates of growth during the juvenile successions; each increase has been found to correspond with a well-defined color phase (Juhn, 1937), and this was taken to indicate a direct participation of the rate of growth in the determination of pigment pattern.

In the F_1 males of crosses between Barred Plymouth Rocks and Brown Leghorns, the juvenile plumage is wholly barred black and white; but, with the adult feathering, red appears in those

portions of barbs lying in the lacy segments of feathers of the dorsal tracts. In the saddle such feathers may show no trace of barring, but, for the most part, the apex is barred black and white, and the feather base is colored. When identified individual follicles were followed for feather succession, a random oscillation was found between barred and partially barred or nonbarred feathers, which suggested that one or the other pattern was laid down with reference to some variable morphogenetic factor (Juhn, 1933). Most recently it has been found that, when the metabolic level of such hybrids is depressed by placing the birds upon a continuous thiouracil diet, barring will appear in the basal colored sections of the exceptional feathers (Juhn, 1945). It may be added in parenthesis that in thyroidectomy, whose effects the drug tends to duplicate, the usual structural changes follow in the feathering of the Barred Plymouth Rock, but the characteristic alternation between black and white bars continues (Parkes and Selye, 1937; Chu, 1940).

In the hybrid cockerel, a bird of somewhat similar genetic constitution as these hybrids, the early juvenile plumage was for the most part also barred, although it contained some red-feather regions—and this in greater area on the right side, which imparted the obvious asymmetry. In the later phases of development of the cockerel there was a distinct tendency toward the formation of red feathers or feather segments at the expense of barred black-and-white plumage elements, and this process was accelerated in the side having initially more red, the right. The plumage potencies for black-and-white barring thus are seen to be determined at lower developmental levels, such as characterize earlier periods of growth of the individual; in later growth phases with accelerating developmental rates, determination of the alternative potency for solid red increased markedly at the expense of barring.

The left side of the bird, where, from the first, barring predominated, may therefore be taken as having lower rates in its physiological processes than the right side, and this is also indicated in the slower onset of molt on the left side (see Tables 2, 3, and 4).

These observations show that in the hybrid cockerel the fundamental basis for the development of the lateral asymmetry resided in a difference in the timing of the general transition from barred black-and-white to solid red feather pigmentation between the two sides of the body, the left always showing a lag. As the cockerel entered its later, more rapid phase of growth, the relative asymmetry of its feathering became accentuated, showing that this initial differential simultaneously increased in steepness. Whether the entire plumage of the bird following later successive molts would finally have become represented as red, with a resulting loss of the visible asymmetry, it is, of course, impossible to say.

In explanation of the differential in the relative speeds of the biological processes which distinguish the two sides of the body of the hybrid and which are seen to be fundamental to the hemilateral asymmetry shown by this bird, two alternative interpretations may be advanced. It is well known, in the first place, that the rate of feathering differs between certain breeds and that slow feathering behaves as a Mendelian dominant (Warren, 1925). Mean axial rates of feather growth may possibly be assumed to vary with rate of feathering and to be determined by the same genes. It could further be assumed that at first cleavage of the hybrid a segregation occurred, causing all descendants of the left daughter cell to lack one gene for fast feathering and, accordingly, being distinguished by lesser

velocities in feathering and in feather growth rates.

However, an organismic asymmetry is a normal attribute of birds: it finds numerous expressions of which only a few can be dwelt upon here. In the embryo the elegant experiments of Rawles have shown the superior developmental capacity of explants of the left side of the blastoderm as compared with the right (1936, p. 308); and the interesting observations of Landauer (1940) on malformations of embryos from eggs of selenium-treated hens show that the asymmetry in the percentage of parts affected may find an opposite expression in different organs, being highest on the left for eyes and wings and on the right for the legs (*ibid.*, pp. 441–42, Table 5).

The asymmetrical persistence of only the left oviduct in the hen is, of course, familiar, but it is perhaps less well known that a similar asymmetry characterizes the Müllerian ducts in the male. Willier and Yuh (1928) found that in experimental modification of incubation the normal processes of regression of the female ducts in the male chick are interrupted and that there is a by far greater number of persistent Müllerian ducts on the left side.

Müllerian vestiges found in the male chick (Juhn and Gustavson, 1932) or in the starling (Kummerlöwe and Froböse, 1930) also are represented in greater lengths on the left than on the right.

In the presence of such diverse manifestations of a constant organismic asymmetry, it is clear that only a slight and perhaps fortuitous accentuation of this normal lateral differential would appear entirely adequate for the development of the asymmetry in the plumage of the hybrid cockerel described in the preceding pages, and this is suggested as the most plausible interpretation.

SUMMARY

Changing feather pattern sequences are described in a hybrid cockerel which displayed a lateral asymmetry in the juvenile and the first adult plumage. As a juvenile the bird was predominantly barred black and white, with traces of red on the left and large red areas on the right. The next feather generation showed that there was an all-over tendency toward the displacement of barring by the red pigment and that this process was more intense on the right. This determined a persistence of the original asymmetry, although of different relative degree.

In interpretation of the quantitative and labile asymmetry shown in the hybrid plumage of this cock, it is assumed that representation of the barred pattern is determined at lower physiological levels, at earlier developmental stages, than is the red pigment and that there was an initial and persistent difference in biological velocities between the two body sides, with the left showing a consistent lag.

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PLATE I

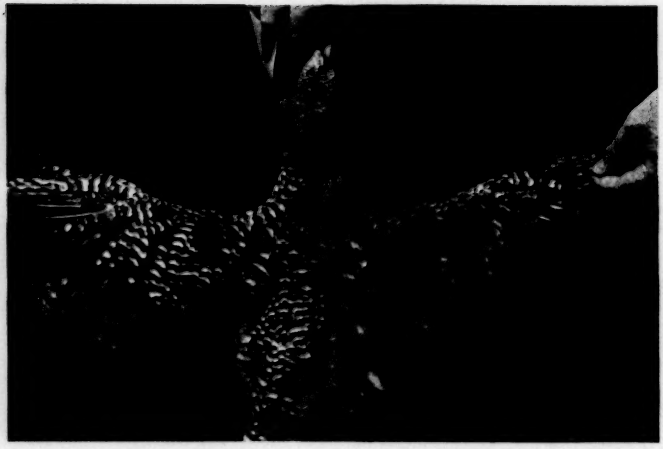

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PLATE I

Color photograph of the hybrid cockerel, M-917. Dorsal view. The photograph, taken while the bird was in the juvenile plumage, shows the presence of barred black and white and of red feathers in both sides of the body, and it also illustrates the quantitative predominance of barred on the left side and of red on the right.

THE RELATION OF THE PLUMAGE-TRACT GRADIENTS TO THE SERIAL LOCALIZATION OF FEATHER PIGMENTS IN A HYBRID COCK[1]

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THE gradients which distinguish the plumage tracts[2] of fowl first aroused attention during earlier experiments on the modification in the female direction of male Brown Leghorn fowl by means of female hormones, or "estrogens," as they are now more commonly designated. Among other results these experiments showed that given feather tracts would respond by a complete or a partial response to varied dosages of the hormone administered subcutaneously (Juhn and Gustavson, 1930) and that similar regional limitations could be obtained within the feather proper (Lillie and Juhn, 1932).

Certain feather tracts proved more favorable than others in the reading of these responses. This was true notably of the breast, where the solid black of the Brown Leghorn male contrasted vividly with the induced marks of female salmon. By relating the size and conforma-

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[2] The individual feather tracts are not compared here. They doubtless arise as embryonic segregates (Lillie, 1929), and their formation precedes the determination of the persistent gradients which distinguish each of these regions throughout life. Differences between tracts may therefore be anticipated to be of a more profound order than between points within a tract.

tion of such marks in this feather tract, caused, for example, by 3-day injections, in longitudinal feather rows of simultaneous regeneration, axes of reactivity could be established (Juhn, Faulkner, and Gustavson, 1931). When such marks were laid down to one side or the other of the shaft of the single feather, they outlined individual asymmetries (Lillie and Juhn, 1932), and, if compared in a series of feathers of simultaneous regeneration along transverse rows, the symmetry relations within the tract were made manifest (Juhn and Fraps, 1934*d*). These observations all indicated persistent and active gradients in the plumage tracts and in the individual feather.

These gradients were investigated more closely in a further series of studies. In some very interesting observations in the embryo, Holmes (1935) found that the order of appearance of the feather-follicle primordia mirrored their later behavior: for example, the first follicle to arise in a future transverse row in each of the breast tracts gave rise later to a feather showing symmetrical marks, while feathers from the follicles later arising to the right and left showed mirror-image marks, as was also true of feathers from follicles of successively greater lateral distance. The future properties of the feathers from follicles of the long axis were also foreshadowed in the extension in the anterior or posterior direction of the first row of embryonic papillae to be laid down.

In defining the gradients in the adult plumage, an approach giving measurable values was found essential. Entire feathers were characterized in their curves of growth, which were constructed from recorded daily or 2-day axial increments.[3] Growth differences, as described by these curves, were taken as a measure of similarity or difference between feathers of given positions. The relative degrees of asymmetry of feathers were estimated from three separate measures. These were, first, the lengths of barbs of corresponding position in opposite vane-halves; second, barb frequencies along comparable right and left surfaces of the rhachis (Juhn and Fraps, 1934*a*, *d*, 1936; Fraps and Juhn, 1936*a*, *b*); and, third, the weights of barbs in opposite vane segments (Fraps and Juhn, unpublished).

Preliminary data were reported by Juhn and Fraps (1934*a*, *b*, *c*, *d*), and detailed studies were published later by Fraps and Juhn (1936*a*, *b*), and by Juhn and Fraps (1936). These papers are to be consulted for details of analysis and procedure; in the present connection it will suffice to outline the relations established in the breast tracts by these methods, as they are the most representative, as well as the most complete. Here it was found that, within each tract, feathers lying along one excentrically located long axis were symmetrical and that this axis formed a center of symmetry, feathers to the right and left showing a directed asymmetry, progressively increasing in degree with distance. Axial growth rates were highest in feathers along the axis of symmetry and decreased with distance in feathers along transverse rows. The degree of this decrease in growth rate was

[3] In much of the early formulation of rate of feather growth, average daily growth increments, taken over comparable periods of growth, served as the criterion of differences in rate of axial growth. In some instances growth was determined from the time the feather was a few millimeters above the surface of the skin until growth was completed. In most cases the data were subject to simple graphic treatment, which sufficiently emphasized the difference in growth rates of compared feathers within or between feather tracts. While more refined procedures might be applied to analysis of the various phases of feather growth, these would merely put into a different form the order of differences, which are quite obvious in the data under discussion.

most marked in feathers located toward and at the lateral margin of the tract (Fraps and Juhn, 1936*b*), an important point in the present discussion, as will appear later. Anteroposterior gradients in axial rates of feather growth had earlier been established in the breast, so that the physiological properties of the co-ordinates were outlined in this tract.

In the saddle, axial rates of growth also tend to be higher in feathers of progressively posterior position in the long axes, but the axis of symmetry in this tract coincides with that of the body. The central feathers are symmetrical, and those toward the lateral margin show a distinct asymmetry of pattern; axial growth rates measured in transverse rows diminish in mediolateral order (Faulkner, 1932). The distinctions in axial rates of growth between feathers of the dorsal tract are not, however, of the same degree as in those found in the breast.

Well-defined progressive increases in axial growth rates of successive feathers take place during the development of the fowl (Juhn, 1937). Velocities proper to the high points of a gradient in earlier periods, therefore, later tend to distinguish its low points; this furnishes a mechanism through which the successive location of distinct feather pigments may, to some extent, be anticipated.

In this paper it is proposed to examine these relations in the successive plumages displayed by a hybrid cock which early gave evidence of a transition from one feather pigment and pattern to another as the bird progressed toward the adult state.

DESCRIPTION

It adds much to the interest of the discussion of the plumages in the cock, M-13760, that the details of the ancestry of the hybrid are known in full. These details are given in the first place, and they are followed by a comparison of successive individual feather generations from follicles selected with reference to their position in the gradients of the breast and saddle, respectively.

In 1938, matings between purebred Barred Plymouth Rock males and purebred New Hampshire females were set up. Barred Plymouth Rock male E-2225 was mated to New Hampshire female H-220. From the progeny of this mating, F_1 male L-3513 was selected. Barred Plymouth Rock male E-2191 was mated to New Hampshire female H-255. From the progeny of this mating, F_1 male L-2584 was selected. Both of the two F_1 males resembled Barred Plymouth Rocks, but they were heterozygous for barring and silver, both of which are sex-linked, carrying one factor for barring and one for nonbarring, one factor for silver and one for nonsilver. They were also heterozygous for the extension factor, *E*.

In 1939, male L-3513 was crossed back to New Hampshire female L-4933. From the progeny of this mating, male M-2963 was selected. This bird was similar to a Barred Plymouth Rock except that the black was replaced by red or gold, as in the New Hampshire. He was heterozygous for barring but homozygous for nonsilver (red or gold plumage color) and for the recessive extension factor (restricting black to wings, tail, and hackle). Male L-2584 was crossed back to New Hampshire female L-5118. From the progeny of this mating, female M-459 was selected. This was a Columbian-patterned bird, being white except for black in the hackle, wings, and tail. She was homozygous for the recessive extension factor and carried the factors for the sex-linked silver and nonbarring. The bird had a pronounced gold or reddish cast, especially over the back and on the

wings; this first appeared at about 4 months of age. Male M-2963 and female M-459 were sire and dam, respectively, of the hybrid male M-13760. This bird was hatched October 9, 1940, and on April 1, 1941, the date at which the first observations and pluckings were made, it still showed considerable juvenile feathering, notably in the saddle, where the transition from one plumage stage to the other proceeds more slowly than in the breast.

I. THE SADDLE

At the initial plucking, six adjacent transverse rows were removed from the left side of the saddle. These rows were designated *A*, *B*, ... *F*, in antero-posterior order. Each row commenced with and included the central feather and all others to the lateral margin. The central feather received the notation 0, or in the first row *A*, 0-*A*. The feathers of next adjacent lateral position in this row followed as 1-*A*, 2-*A*, and so on, to 8-*A*, at the extreme left extension of the row. All other feathers were identically identified for location and row. As the feathers were removed, they were mounted in this order of position, separate cardboard sheets appropriately numbered and also carrying the date of plucking having been prepared for each row. This arrangement facilitated comparisons between feathers of any one point of the gradient and also, of course, made possible a direct confrontation of precursor and successor feathers from any given position.

The original series of saddle feathers plucked contained a large number of early juvenile feathers. These were often rounded in shape and generally marked in the apical third of the feather vane by an irregular narrow white bar followed by a wide black bar and sometimes by a second edging of white. Other juvenile feathers, while still short, were more pointed in shape, having a distinct red barring with a little black near the rhachis upon a white ground. Toward the fluff such feathers showed large black and red segments; the fluff itself was gray, with occasional black marks at the rhachis, the marginal barb tips being red. In the latest of the juvenile stages represented at this date, the feathers commenced to approach the adult appearance, and, although falling short of the ultimate lengths, they had developed the lacy margin which characterizes the final development in this region. When such feathers were of central location, the apical third or half of the vane was barred red and white in the solid portion and a deep orange at the fringed margin. Basally, there followed a wide white bar and then a red zone with a black mark at the rhachis, which mark usually continued into a small white mark. Feathers of this same stage, but located at the margins of the tract, also contained some orange in the lacy portions of the feather vane, but the entire solid core was white; black marks edged with red appeared just above the fluff. Feathers of mid-position in the rows were intermediate in appearance.

The feathers replacing this series were individually collected and mounted in the same manner, $3\frac{1}{2}$ months later. A comparison of the two generations showed a complete absence of the barred pattern which had been developed in a good number of the early juvenile feathers. In general, the tendency was toward the diminution in the representation of the red pigment; this proceeded in distinct mediolateral order. Feathers of positions 0, 1, and 2 possessed considerable red and resembled feathers of the latest stage and in this location in the preceding series. Feathers of more lateral position showed progressive increases in

the area of the feather vane occupied by white. Feathers of the margin were almost completely white, with some faint orange at the tip and with small asymmetric black marks just above the fluff and vestiges of red in the barb apices of the fluff proper (see color photographs in Pl. I).[4]

Summary.—A comparison of successive series of saddle feathers grown from identical follicles showed red-and-white barring and solid red, with some black in the early stages. This barring disappeared completely in later stages, and all the area occupied by the red pigment tended to be reduced or to be completely replaced by white. The displacement took place more rapidly in feathers of lateral, than in feathers of central, position.

2. THE BREAST

The feathers were removed and mounted as described for the saddle. At the time of the first observation, ten adjacent transverse rows were plucked from the left breast tract. These rows were identified as *A*, *B*, *C*, ... , *J*, in anteroposterior order. In each row the individual feathers were numbered, as had been done for the saddle, in accordance with a scheme set up in other, earlier observations (Juhn and Fraps, 1934*d*). The separate rows of the breast tracts form inverted V's in their natural position. In the Brown Leghorn there are nine follicles in a transverse row; No. 1 is near the mid-line of the body, No. 9 is at the lateral margin of the tract, and No. 6 is at the peak. Follicles of this No. 6 position lie on the axis of symmetry of the tract. For purposes of comparison, this number has been kept a constant; and where, as, for example, in the Barred Rock, there are eleven follicles to a row, the additional two follicles are numbered 9′ and 1′, respectively. In the hybrid cock there were also eleven follicles to the row, and this system of notation was used.

Some of the early juveniles had not been molted in the first series of feathers. These feathers were irregularly barred black and white, with a dusting of red in positions near the mid-line. Feathers of the lateral margin were red, with a little black and white. The more generally represented later stage also contained red, black, and white pigments. These feathers, when located in the anterior rows, *A*–*F*, and especially from positions 9′, 9, and 8, showed much red. Feathers of positions 7 and 6 exhibited much less of this red pigment, and it was reduced to traces along the edges of the vanes of the black-and-white feathers of medial location, Nos. 5–1, 1′. In the feathers of the more posterior rows, the red pigment became progressively restricted to feathers of the lateral margin and finally appeared only in traces even in this location.

The examination of the individual feathers showed that feathers of the 9′ position were asymmetrical, red in one vane-half and black in the other in the anterior rows and with an asymmetric irregularity of the black-and-white barring in the posterior rows. Feathers of position 9 were usually solid red, with occasional asymmetric black marks. Feathers of positions 8, 7, and 6 tended to exhibit more or less symmetrical white-tipped black spangles followed by white with dashes of red; these spangles again disappeared in the feathers of medial positions (see color photographs in Pl. II).

A second plucking was made 2 months and 11 days later, and a last regeneration was collected 2 months after this time; the description given is of

[4] The color and other photographs for this paper were prepared by Mr. John Spurbeck, to whom the authors wish to express their appreciation and indebtedness.

the final records. In this series feathers of the lateral margin in the most anterior rows continued to show red, black, and white pigments, but all the other feathers had become pure black and white. The black and white pigments combined to form different patterns at separate locations along the transverse axes. Feathers centrally located in a transverse row showed a distinct black spangle with a small white tip; this spangle was fully symmetrical in feathers lying along the axis of symmetry of the tract. In feathers immediately to the right and left of this axis, the spangle became slightly asymmetrical, and the pattern shown in one of this pair of feathers was the mirror-image of the other. The degree of this asymmetry increased sharply with the distance of each feather from the center of symmetry and in more rapid order in the direction of the lateral margin of the tract, so that the spangle became distorted, leading finally to an almost complete separation of black and white in the two vane-halves. These all-over transverse pattern series were found to vary in degree, although not in principle, with the position of the row in the long axis (see Pl. III).

Summary.—A comparison of successive series of breast feathers grown from identical follicles showed in the early juvenile stage an irregular black-and-white barring, with dashes of red, and solid red, with some black and white in feathers of the lateral margin. In later stages this barring disappeared. Red continued to be laid down extensively only in feathers of the lateral margin of the more anterior rows. Feathers of other positions became black and white and a distinct symmetrical black spangle developed in feathers along the axis of symmetry and of slightly lesser symmetry in the immediately adjacent feathers.

DISCUSSION

The examination of the separate patterns which were successively developed in the plumages regenerated by the hybrid cock clearly demonstrate the participation of the feather-tract gradients in these processes. Specific patterns (barring) and pigments (red predominating), which characterized the earlier juvenile feathers, continued to be laid down in later stages only in those points of the gradients distinguished by the persistence of slower axial growth rates—in the breast, feathers from more anterior rows and from the lateral margin; in the saddle, feathers from anterior rows and of central position.

The spangled pattern, which was not an initial attribute but which was formed in feathers of later regeneration in the breast, beautifully reflected the action of the symmetry relations which had been earlier established in this tract. The spangle ultimately developed in feathers from the axis of symmetry was symmetrical. Asymmetry of the spangle increased with distance of feather position and in a greater degree in the series of feathers characterized by a rapid decrease in axial rates of growth, from positions approaching and at the lateral margin.

These relations all support the earlier interpretations advanced elsewhere, that in purebred particolored fowl (Juhn, 1937, 1938), as well as in the hybrid (Juhn, 1933), the determination of the regional and temporal expression from the genetic diversity of given pigments and patterns follows from the physiological levels in effect at that time and at that place in the gradients of the individual feather tracts.

An interesting example of changing pattern is the appearance, in later juvenile plumages, of the spangle in the Silver

Spangled Hamburg. Successive feather series were described by Dunn and Landauer (1930), who concluded (*ibid.*, pp. 44–45) that the genetic constitution of the follicle is only one item in the reaction which produces the pattern and that there are other components which appear to be released or activated at given stages in development.

Lillie and Juhn (1937, 1938), however, have found that, in male adult fowl, changes in pattern also occur and that these are controlled by properties of growth then in effect in the feather. During formation of the after-feather, fission of the ventral triangle takes place. One vane-half of the main feather and one vane-half of the after-feather derives from one each of the two daughter triangles, and it is these two vane-halves which exhibit correspondence of pigment marks, etc.

The hybrid cock described in the preceding pages provided unusually favorable material for the recognition of the changing orders in the gradients as these were reflected in the pigments and patterns laid down successively in feathers regenerated by follicles of relevant position. Such relations could pass unperceived in self-colored plumages and be obscured in the presence of an all too great complexity of the genes for diverse pigments and pattern. However, it is probable that, once the topography of the gradients is clearly in mind, their action may also be readily recognized in numbers of wild and domestic plumage patterns.

A final point which is of very considerable interest and one which deserves further recognition and study is the relation of all-over accelerations to the individual growth properties of the feathers. Are there at such times increasing differences in axial growth rates between feathers along the co-ordinates, so that the initial slopes of the gradient become steeper, and are there also simultaneously progressive accentuations in the relative degrees of asymmetry of feathers lying along the transverse axes?

SUMMARY

The feather patterns successively displayed in early juvenile and later plumage stages in a hybrid cock of known ancestry were studied in relation to the gradients in the breast and saddle. This bird gave evidence of a transition from black, red, and white marks and irregular bars to a distinct black spangle on a white ground in the breast, and from irregularly barred red and white with some black toward solid white in the saddle.

Through appropriate pluckings, feathers were successively regenerated along the gradient co-ordinates, and it was established that pigments and patterns represented in early stages and which are distinguished by lower developmental velocities continued to be laid down in later stages only in previously established low points of the gradients. Similarly, development of the spangled pattern took place in the frame of symmetry relations which had been earlier defined in other birds.

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PLATE I

The color photographs give a comparison of the patterns displayed in juvenile and in adult feathers of comparable position in transverse feather rows of the left saddle.

Upper row, feathers plucked on April 1, 1941. From left to right the feather positions are *D*-2, *E*-4, *B*-5, *B*-7 (see text).

Lower row, feathers plucked on July 15, 1941. From left to right the feather positions are *F*-0, *F*-2, *F*-4, *F*-6 (see text).

PLATE II

The color photographs give a comparison of the patterns displayed in late juvenile and in adult feathers of comparable position in transverse rows of the right breast tract.

Upper row, feathers plucked on April 1, 1941. From left to right the feather positions are *A*-9′, *A*-9, *A*-8, *A*-7, *A*-5 (see text).

Lower row, feathers plucked on August 12, 1941. From left to right the feather positions are *A*-9′, *B*-9, *A*-8, *A*-7, *A*-5 (see text).

PLATE III

The photographs show the development of pattern within the frames of the symmetry relations obtaining in the breast tracts. From left to right the feather positions are *G*-8, *G*-7, *G*-6 (feather follicle lying in the axis of symmetry, see text), *G*-5, *G*-4.

PLATE I

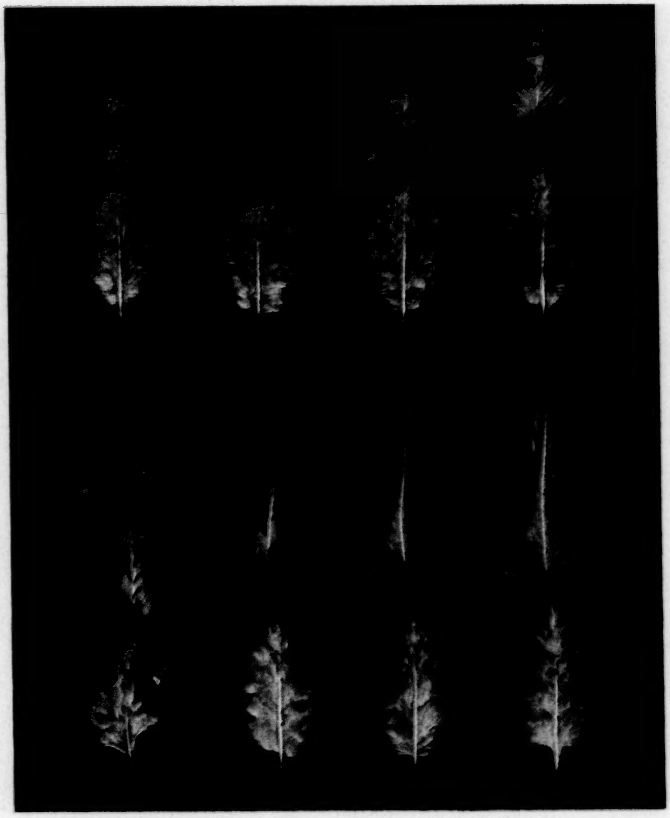


PLATE II

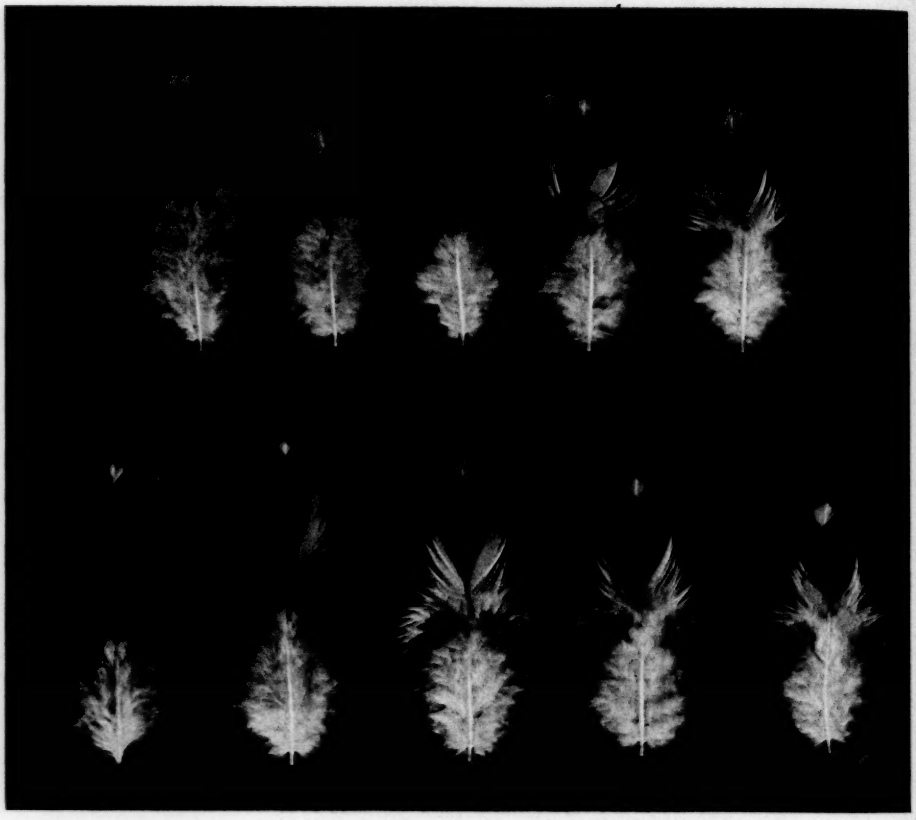
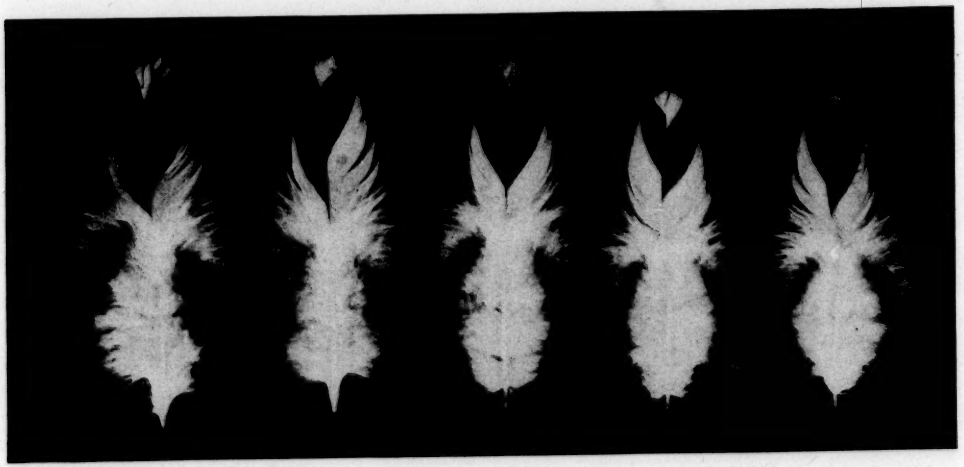


PLATE III



PHOSPHORYLATION IN THE FROG'S EGG

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A PRIMARY aim of developmental physiology is to demonstrate the manner in which metabolic energy is transferred to the specific form-building activities of the developing embryo. The present attack on this problem stems from the study (Barth, 1946) of the block to metabolism in gastrulae resulting from the cross, *Rana pipiens* ♀ × *R. sylvatica* ♂ (Moore, 1941). The former author found that, although hybrid and normal gastrulae are characterized by the same respiratory quotient, both oxygen consumption and lactic acid production are stabilized in the hybrid "permanent gastrulae" at the early gastrular level, both processes failing to show the increases characteristic of normal *pipiens* during subsequent days. Interested in the nature of this simultaneous block to metabolism and development, Barth suggested a deficiency in, or malfunction of, some "coupling" mechanism common to both respiration and glycolysis. For such a coupling mechanism a pattern was available in the form of the energy-rich phosphorylated intermediaries of muscle that *accumulate* and transfer glycolytic energy to myosin. The present paper represents an attempt to determine whether energy-rich phosphate compounds play a role in the transfer of energy to normal developmental processes, especially gastrulation, and whether this energetic coupling is disturbed in the hybrid gastrulae.

The role of phosphate compounds in amphibian development has not been investigated systematically. Plimmer and Kaya (1909) reported no inorganic phosphate at "the beginning of development" and only traces at "the end" (? hatching). As will be pointed out in the description of extraction procedures, their low inorganic phosphate values may be attributable to failure to free the extract of glycogen, with which inorganic phosphate appears to be closely associated in the amphibian egg. Harris (1944) found a phosphoprotein phosphatase in ovarian eggs, which he suggested might be involved in the transfer of inorganic phosphate from the yolk to the glycolytic systems of the egg. Brachet and Chantrenne (1945) found in preliminary experiments a marked synthesis of adenosine-tri-phosphate (ATP) at gastrulation and neurulation, especially in morphogenetically active regions; but the significance of this finding remains to be clarified.

The use of specific inhibitors of phosphorylation, such as iodoacetate and fluoride—a conventional technique for attacking the phosphate cycle—could not be applied to the study of gastrular metabolism, since the senior author (Barth, unpublished) found that penetration of these inhibitors was uncertain. It would be, on the other hand, naïve to assume that, if energy-rich phosphate compounds intervene in development, their concentrations will vary with the developmental activity in progress. If the phosphorylation cycle is functioning smoothly, its component intermediaries, in steady state, should not accumulate,

despite the changing demands of the developmental system. The *rate* of turnover might vary from stage to stage, but rate changes could not be established by direct quantitative analysis of intermediaries. A more fruitful approach, therefore, would appear to be the finding of a means whereby phosphorylation may be thrown "out of gear" with respiration, so that the latter process no longer can supply the energy necessary to keep such compounds as ATP in the phosphorylated condition. Such a dislocation of energy production from energy transfer should be reflected, then, in a quantitative redistribution of phosphorylated intermediaries. Should such a technique also result in specific embryological disturbances, it might offer some insight into the nature of the coupling between metabolism and development.

The principal device chosen for the present experiments was the introduction of anaërobiosis. Sodium azide was used in a few experiments, and hybrid gastrulae provide a "natural" instance of the disturbance in energy transfer. It was known (Barth, 1946) that gastrulation would proceed, though at a slower rate, in the absence of oxygen at the expense of a lactic acid debt. The initial question for the present study therefore became: Does the shunt from aërobic to anaërobic energy production result also in a quantitative redistribution in the normal gastrula of energy-rich phosphate compounds such as ATP?

METHODS

BIOLOGICAL

Eggs were obtained by pituitary injection of *R. pipiens* from Vermont that had been stored in the laboratory at 3°–5° C. for less than 2 weeks. Methods of obtaining eggs and of fertilization, care of the eggs during development, and removal of jelly layers have been described in full elsewhere (Barth, 1946). A modified 0.1 Ringer's solution, lacking bicarbonate and phosphate, was used throughout the experiments.

After the desired number of eggs had been freed of jelly, they were examined under a binocular microscope, any abnormal or injured eggs being discarded at this time. Eggs thus known to be in good condition were counted out into Stender dishes, 100 per dish.

USE OF WARBURG FLASKS AS ANAËROBIC CHAMBERS

The eggs were pipetted from the Stender dish into a graduated 15-cc. centrifuge tube, and the excess 0.1 Ringer's was drawn off, to leave a volume of eggs plus 0.1 Ringer's amounting to 2.8 cc. These egg suspensions were poured into clean Warburg flasks with one side-arm. Potassium hydroxide was added to the center well or H_2SO_4 to the side-arm, depending upon whether oxygen consumption or carbon dioxide production was to be measured. When the flasks had been attached to the manometers in an 18° C. constant-temperature bath, an equilibration period of 10–15 minutes with shaking was introduced. In those flasks provided with gaseous nitrogen instead of air, the nitrogen, before entering the flasks, was passed over heated copper wire and bubbled through a wash bottle suspended in the bath. A record of oxygen consumption was kept to ascertain the degree of anaërobiosis.

After the desired time had elapsed, the flasks were removed from their manometers, and the KOH was washed out. The eggs were transferred to Stender dishes of fresh 0.1 Ringer's solution, where their stage and condition could be examined quickly. They were then

pipetted into pre-chilled 12-cc. heavy-duty Pyrex centrifuge tubes for phosphate analyses. The analyses, therefore, are of phosphate in eggs only and not of solution. Preliminary tests showed that no phosphate diffused from the eggs during the longest period (22 hours) used.

CHEMICAL

I. EXTRACTION OF ACID-SOLUBLE PHOSPHATES WITH TRICHLORACETIC ACID

The extraction method of Lepage and Umbreit (1945) was used with certain modifications. After considerable investigation we were able to obtain, from the amphibian egg, trichloracetic extracts free of fat, protein, and glycogen, all three of which, if present in the final extract, interfere, by producing turbidity, with the colorimetric determination of phosphate. Lipids could be eliminated by chilling all reagents and tubes to 3°–5° C. during the extraction and by centrifuging in the refrigerator—procedures which also minimized the possibility of phosphocreatine breakdown. Extracts free of interfering proteins were obtained only after the high optimum concentration of trichloracetic acid was discovered. Glycogen was removed by alcoholic precipitation, although other methods of removing this polysaccharide are being examined (mercuric sulphide precipitation and ultra-centrifugation).[1]

The method finally settled upon for these experiments began with transfer of 100 eggs to the pre-chilled centrifuge tube (an ice-bath being used to avoid the necessity for working in a cold room), withdrawal of excess 0.1 Ringer's, and chilling for 5 minutes. One cc. of ice-cold 75 per cent trichloracetic acid was added, and the eggs were homogenized by moving a cold stirring-rod up and down in the tip of the centrifuge tube. The eggs, trapped between the rod and the sides of the tube, were crushed to a brei within 60–90 seconds. Microscopic examination showed the same degree of homogenization in breis obtained by this method as in breis made with Ten Broeck tissue-grinders. Replaced in the ice-bath, the tubes were chilled for 8 minutes, after which 7.5 cc. of ice-cold distilled water were added and the homogenate was redispersed with the stirring-rod. After 2 minutes' chilling, homogenates were centrifuged in the refrigerator at 2,500× gravity for 3 minutes. The still-cold supernatant fluid was decanted into a second chilled 12-cc. heavy-duty Pyrex tube. The residue (now nearly free of acid-soluble phosphates) was washed with 2 cc. of cold 7.5 per cent trichloracetic acid, in which the residue was redispersed by means of a narrow stirring-rod. After 2 minutes' chilling, the second extract, obtained by centrifuging for 3 minutes, was added to the original extract.

At this point the total extract, still cloudy with glycogen, was mixed thoroughly by placing a square of Parafilm[2] over the tube and inverting repeatedly. The extract was divided equally between two tubes in preparation for the precipitation of glycogen. This latter was accomplished by adding an equal volume (*ca.* 5.5 cc.) of cold 95 per cent redistilled ethyl alcohol, mixing, and chilling for 10 minutes. The glycogen was thrown down by 10 minutes' centrifuging in the cold at 1,950×gravity. The now clear supernatant fluid was decanted into 15-cc. Pyrex tubes, to which had already been added 1 cc. of 1 N NaOH and 2 microdrops of phenolphthalein. Neutralization

[1] After ultra-centrifugation to remove glycogen, the supernatant fluid showed little or no phosphate, indicating a glycogen-phosphate complex.

[2] Parafilm, when cut into squares which retain until used the waxed-paper backing, provides a disposable cap, superior, since there is no loss of fluid, to rubber stoppers for mixing.

was completed by adding, drop by drop, 0.1 N NaOH or 0.1 N HCl to a faint pink with phenolphthalein (pH about 8.2).

II. CALCIUM PRECIPITATION OF INORGANIC PHOSPHATE, ADENYLPYROPHOSPHATES, AND FRUCTOSE-DI-PHOSPHATE

Since we were interested, first, in following the inorganic phosphate–ATP balance of the eggs, it was decided to circumvent the longer barium-precipitation method by the direct calcium precipitation of these compounds, according to Lardy *et al.* (1945). One-fifth volume of 10 per cent $CaCl_2$ saturated with $Ca(OH)_2$ was accordingly added to the neutralized extracts, mixed, and allowed to precipitate at room temperature for 10 minutes. The heavy precipitate of calcium salts was centrifuged down thoroughly and the supernatant fluid discarded. The precipitate, washed by redispersal in 20 per cent calcium reagent, was recentrifuged.

III. DETERMINATION OF INORGANIC PHOSPHATE AND ADENYLPYROPHOSPHATES

A. *Hydrolysis.*—The calcium precipitates were sorted into the pairs that resulted from division of the original trichloracetic extract, for 7-minute hydrolysis in 1 N HCl at 100° C., and for 0-minute hydrolysis, the latter giving the true inorganic phosphate value. Two cc. of 2 N HCl were added to all tubes, and the precipitates were dissolved by tapping the tubes. Two cc. of distilled water were mixed with the acid to bring the concentration to 1 N. Tubes to be hydrolyzed were capped with glass deflamators and transferred to a water bath at 100° C. for 10 minutes (3 minutes being needed for tubes to reach 100° C. and 7 minutes for hydrolysis). The tubes were then plunged into a water bath at room temperature, the unhydrolyzed tubes were added to the rack, and all were allowed to equilibrate to below 25° C. for 5 minutes.

B. *Inorganic phosphate determination.*—The Fiske-Subbarow method as described by Lepage and Umbreit (1945) was used to determine the 7-minute and the 0-minute inorganic phosphate content of corresponding pairs of tubes. Since temperature, acid concentration, and time are all critical for color development, the following precautions were observed: All tubes were allowed to reach the same temperature. Acid and water for hydrolysis had been measured carefully. The ammonium molybdate and reductant prescribed by Fiske-Subbarow were added to each tube individually. The whole, diluted to 10 cc., was mixed, and the starting-time of color development was recorded for each tube separately. The resulting blue solution was transferred to a colorimeter tube and read, after 10 minutes' color development, in a Klett-Summerson photoelectric colorimeter with a 660-mμ filter.

Duplicate 4-cc. samples of standard KH_2PO_4 solution, containing 10 μgm. of phosphate per cubic centimeter, were carried through the entire extraction and precipitation procedure, including splitting for 7-minute phosphate and 0-minute phosphate determinations. All colorimeter readings, after subtraction of 10 K-S units for the reagent blank, were referred to the corresponding standard for conversion into micrograms of phosphate. Labile phosphate thus was determined by the difference between the inorganic phosphate content after 7-minute acid hydrolysis (referred to hereafter as "total phosphate") and that of the unhydrolyzed member of the pair (true inorganic phosphate). The method did not enable us to distinguish between ATP and adenosine-di-phosphate (ADP). This distinction, however, seems unessential at so preliminary a stage in the

analysis of gastrular phosphorylation. Fructose-di-phosphate, also present in the calcium precipitate, is only 26.5 per cent hydrolyzed under the condition simposed and since, further, the fructose content was found to be very low, probably may be discounted as a major source of the Δ7-minute phosphate.

Because the absolute values vary from one batch of eggs to another, it has been found convenient sometimes to express the inorganic phosphate value as percentage of total (i.e., 7-minute hydrolyzable *plus* inorganic). This device was not applied, however, when the aërobic and the anaërobic total phosphate values differed significantly within an experiment.

EXPERIMENTAL

AËROBIC DEVELOPMENTAL SERIES

Eggs submitted to anaërobic conditions at any stage continue to develop for a time that depends on the ratio between the energy available from lactic acid production and the energy demands of the embryo for maintenance and development (Barth, 1946). If an influence of anaërobic conditions upon energy-rich phosphate content is to be demonstrated, the question of whether these phosphates vary from stage to stage, even under *aërobic* conditions, becomes a critical point.

Series of analyses were made on eggs from the same clutch at seven successive developmental stages. As soon after fertilization as the jelly had swollen, eggs were cut into small groups and divided between a number of finger bowls to prevent crowding. The eggs developed at temperatures ranging from 15° C. to room temperatures and were removed from jelly and analyzed in duplicate at the following stages: early cleavage (Shumway's [1940] Stages 4–7); late cleavage (Stage 8); blastula (Stage 9); early gastrula (Stage 10); mid-gastrula (Stage 11); late gastrula (Stage 12); and neural plate (Stage 13).

The results of three entire and seven partial such aërobic series are shown in Table 1 for the total, inorganic, and Δ7-minute fractions, converted to micrograms of phosphate per 100 eggs.

TABLE 1

AËROBIC DEVELOPMENTAL SERIES

Summary of data on total, inorganic, and Δ7-minute phosphate fractions, expressed as micrograms per 100 eggs. Each value represents average of two duplicate analyses.

Stage	Phosphate (Micrograms/100 Eggs)		
	Total	Inorganic	Labile
Early cleavage (Stages 4–7)	41.4	22.2	19.2
	45.3	25.9	19.4
	38.4	21.2	17.2
	54.3	26.7	27.6
	50.2	26.2	24.0
	38.4	25.2	13.2
Mean	44.7	24.6	20.1
Late cleavage and blastula (Stages 8–9)	41.1	28.1	13.0
	46.9	23.4	23.5
	38.6	18.2	20.4
	45.5	27.4	18.1
Mean	43.0	24.3	18.7
Early and middle gastrula (Stages 10–11)	39.1	29.8	9.3
	48.0	22.3	25.7
	55.4	26.1	29.3
	48.4	31.2	17.2
Mean	47.7	27.4	20.3
Late gastrula and neural plate	41.6	25.5	16.1
	55.3	21.0	34.3
	48.8	26.6	22.2
Mean	48.6	24.4	24.2

That there is considerable variation in absolute values between different batches of eggs will be apparent both in this set of experiments and in all successive tables. This variation may be at-

tributed, in part at least, to the size of the eggs, a fact established by correlation of total nitrogen, kindly determined for us by Dr. John Gregg, with absolute values for total and inorganic phosphate. Since such a variation is based on a non-random factor intrinsic in the biological material, one has the alternatives of presenting each series separately by single egg clutches or of assuming that the mean values of all series taken together will have absorbed equally from stage to stage this variability based on egg size. The latter expedient has been resorted to.

The data here presented show no change in the inorganic phosphate fraction between early-cleavage and neural-plate stages, unless the temporary small rise at gastrulation is considered significant. This transient increase of inorganic phosphate, if real, comes from an ester outside the labile fraction, since the total value rises simultaneously. The extrinsic inorganic phosphate is absorbed apparently between gastrulation and neural plate by the labile fraction, which at Stage 13 shows an increase, unaccompanied by change in total phosphate (cf. Brachet's table, p. 478, which shows a synthesis of ATP between gastrula and neurula stages).

One may conclude from this series of experiments that the ratio between inorganic and labile phosphates remains relatively constant throughout early development and that any experimentally produced changes in these fractions may not be attributed to autonomous stage-to-stage fluctuations.

THE EFFECT OF 22-HOUR ANAËROBIOSIS ON THE DISTRIBUTION OF PHOSPHATE BETWEEN LABILE AND INORGANIC FRACTIONS DURING GASTRULATION

Early gastrulae subjected to an atmosphere of nitrogen in the Warburg flasks continue to develop, though at a retarded rate, so that, after 20–24 hours at 18° C., they are found to have reached Stages 11–11+, as compared to Stages 12–12+ for the aërobic controls. At the end of this period aërobic and anaërobic gastrulae were analyzed for their content of inorganic and total phosphate. Although in the results (Table 2) the usual variability in absolute amounts is apparent, a striking rise of inorganic phosphate in the anaërobic gastrulae is seen ($P = <0.01$). When all the experiments are considered together, with regard to total phosphate, no significant difference appears between aërobic and anaërobic gastrulae ($P = 0.7–0.8$). Upon closer examination it is seen, however, that the experiments fall into two categories: those in which the increase in inorganic is accompanied by an increase in total phosphate, and those remaining in which either no change or a slight decrease in the total fraction occurs. It would appear, therefore, that, although labile phosphate always breaks down under anaërobic conditions (the increase in inorganic phosphate always exceeds the increase in total), the breakdown of ATP may be supplemented by hydrolysis of an ester not included in the fraction that we studied. The nature of this extrinsic ester and the factors conditioning its participation or lack of participation during anaërobic metabolism remain to be established.

Upon return of the gastrulae to air after a long period in nitrogen, development is resumed, and oxygen consumption rises sharply, even exceeding for a time that of aërobic controls at the comparable stage (Barth, unpublished). What effect does the introduction of air after prolonged anaërobiosis have upon the inorganic and labile phosphate content of gastrulae? Table 3 indicates that readmission of air leads very shortly (within 2–3 hours) to a rephosphoryla-

TABLE 2

TOTAL, INORGANIC, AND Δ7-MINUTE PHOSPHATES OF THE GASTRULA AFTER 22 HOURS' ANAËROBIOSIS

Each value, expressed as micrograms per 100 eggs, represents the average of two duplicate analyses.

Date	Total	Inorganic	Labile	Percentage Increase of Inorganic in N_2	Percentage Change of Total in N_2
	Air				
11-20	67.7	32.1	35.6	.....	.....
11-21	60.3	30.4	29.9	.....	.....
11-27	70.5	32.9	37.6	.....	.....
1-2	48.7	31.3	17.4	.....	.....
1-6	49.6	27.9	21.7	.....	.....
1-8	41.8	22.1	19.7	.....	.....
1-10	42.5	20.1	22.4	.....	.....
3-29	31.4	19.2	12.2	.....	.....
4-1	47.7	18.5	29.2	.....	.....
4-26	53.0	22.6	30.4	.....	.....
1-18	36.8	19.2	17.6	.....	.....
1-22	47.3	22.8	24.5	.....	.....
1-24	40.7	18.8	21.9	.....	.....
Mean	49.1	24.5	24.6	.....	.....
	Nitrogen				
11-20	67.0	44.6	22.4	39.0	− 1.4
11-21	59.2	40.5	18.7	33.3	− 1.7
11-27	67.8	48.3	19.5	46.8	− 3.8
1-2	48.2	32.8	15.4	5.0	− 1.3
1-6*	53.2	33.9	19.3	21.5	+ 7.3
1-8*	43.4	38.2	5.2	72.8	+38.3
1-10*	43.8	27.5	16.3	36.8	+ 3.6
3-29*	43.9	28.3	15.6	47.4	+39.9
4-1*	55.2	36.1	19.1	95.2	+15.7
4-26	47.7	29.3	18.4	29.7	−10.0
1-18*	44.7	32.2	12.5	67.8	+21.5
1-22*	55.3	35.7	19.6	56.6	+16.9
1-24	37.8	32.8	5.0	74.4	− 7.2
Mean	51.3	35.4	15.9	48.2	.....
	t=0.38 P=0.7–0.8	t= 3.32 P=<0.01	.....	.....	.....

* Experiments marked with an asterisk are those showing anaërobic increase in total phosphate.

tion of the labile phosphate, which was hydrolyzed during the 22-hour period of low energy output resulting from anaërobiosis. Rephosphorylation takes place rapidly on admission of air, so that some of the variability in labile phosphate in Table 2 may be the result of recovery during the time when the manometers were taken apart and during examination and transfer of eggs prior to homogenizing. In the experiments in Table 3 the eggs were chilled immediately after removal from nitrogen and homogenized, which accounts for the lower values of labile phosphate obtained after 22 hours of anaërobic conditions. Examination of Table 3 at 4 hours reveals, moreover, that there is even "overrecovery" during the 3–8 hours after readmission of air. An actual *synthesis* of labile phosphate with corresponding decrease in inorganic phosphate appears during this period, by comparison with controls that developed in air throughout. The possible correlation of this "overrecovery" with the simultaneous "overshoot" in respiration will be discussed in a later section.

The gastrula is thus shown to possess enzyme systems for the transfer of phosphate to and from ATP. The state of relative phosphorylation or dephosphorylation of this energy-rich compound would appear to depend upon the quantity of energy available from either respiration or glycolysis. The latter process seems less efficient in the task of keeping the phosphorylated compound in the amphibian gastrula at a high concentration.

EFFECT OF 22-HOUR ANAËROBIOSIS ON THE DISTRIBUTION OF PHOSPHATE BETWEEN LABILE AND INORGANIC FRACTIONS DURING CLEAVAGE

Experiments similar to those reported in the preceding section were made on eggs in early cleavage stages. Such eggs, together with aërobic controls, were subjected to 20–22 hours in nitrogen in the Warburg flasks. The analytical results, presented in Table 4, are in marked contrast to those of gastrular stages (cf. Tables 2 and 4). Whereas gastrulae after 22 hours of anaërobiosis show a 48 per cent increase of inorganic phosphate, cleavage stages show an increment of only 11 per cent. It would appear, therefore, that during cleavage the supply of energy-rich phosphate in the form of ATP remains sufficiently intact, even under anaërobic conditions, to meet the lower energy demands both for maintenance and for development. Examina-

TABLE 3

RATE OF REPHOSPHORYLATION

Recovery, through resynthesis of labile and decrease of inorganic phosphate, after readmission of air to 22-hour anaërobic gastrulae. Results, expressed in micrograms per 100 eggs, shown for duplicate analyses.

22 Hours Air			22 Hours Nitrogen			2 Hours Recovery			4 Hours Recovery			8 Hours Recovery		
Total	Inorganic	Labile	Total	Inorganic	Labile	Total	Inorganic	Labile	Total	Inorganic	Labile	Total	Inorganic	Labile
45.5	28.5	17.0	41.7	37.7	4.0	49.7	33.6	16.1	49.7	28.1	21.6	41.0	25.0	16.0
46.3	29.9	16.4	42.1	37.9	4.2	47.3	31.6	15.7	46.9	27.3	19.6	45.3	26.7	18.6
Mean														
45.9	29.2	16.7	41.9	37.8	4.1	48.5	32.6	15.9	48.3	27.7	20.6	43.2	25.9	17.3

tion of the total and inorganic phosphate values reveals that about half the increase in inorganic phosphate in nitrogen during cleavage stages comes from breakdown of an ester other than ATP, i.e., the anaërobic percentage increase of inorganic is 11.1 per cent, while the total increases 5.3 per cent.

THE RATE OF ATP BREAKDOWN DURING ANAËROBIOSIS AT GASTRULATION

At the end of 22 hours in nitrogen the amount of gastrulation represents only about 60 per cent that of aërobic controls. During this long anaërobic period the inorganic phosphate has increased approximately 50 per cent (Table 2). Anaërobiosis appears to induce an abrupt cessation of gastrulation rather than a gradual retardation spread throughout the prolonged exposure to nitrogen (Barth, unpublished data). Does the increase of inorganic phosphate occur gradually during 22 hours' anaërobiosis, or is this phenomenon also confined mainly to one phase of the over-all period?

Five experiments were made to learn the rate of hydrolysis of ATP in early gastrulae submitted to a nitrogen atmosphere in the Warburg flasks. The resulting mean values, expressed in micrograms of phosphate per 100 eggs, are presented in Table 5. Although the usual variability in absolute values is seen in these data, when inorganic is expressed as percentage of total phosphate, a gradual rise of inorganic phosphate appears as early as 2–4 hours. Grouping the 2- and 4-hour data for aërobic and anaërobic gastrulae gives a number of cases sufficient to demonstrate a statistically significant ($P = <0.01$) increase in inorganic phosphate,

$$\frac{\text{Inorganic}}{\text{Total}} \times 100,$$

even during this short period. The data grouped for 8- and 16-hour anaërobiosis also show a significant increment in inorganic phosphate over both 2–4-hour anaërobic and 2–4-hour aërobic gastrulae. As previously noted, some of the inorganic phosphate is derived from the breakdown of an unknown ester.

When, moreover, the percentage increase of inorganic phosphate is calculated as

$$\frac{\text{Inorganic in } N_2 - \text{inorganic in air}}{\text{Inorganic in air}} \times 100,$$

TABLE 4

TOTAL, INORGANIC, AND Δ7-MINUTE PHOSPHATES OF CLEAVAGE STAGES AFTER 22 HOURS' ANAËROBIOSIS

Development in nitrogen proceeded from Stages 3–6 through Stages 9–10. Each value, expressed as micrograms per 100 eggs, represents the average of two duplicate analyses.

Date	Air				Nitrogen			
	Total	Inorganic	Labile	Per Cent (Inorganic/Total)	Total	Inorganic	Labile	Per Cent (Inorganic/Total)
2-10	35.4	17.4	18.0	49.2	35.7	22.5	13.2	63.0
2-12	48.4	31.2	17.2	64.4	54.0	31.9	22.1	59.1
2-14	45.5	27.4	18.1	61.5	46.6	30.0	16.6	64.5
Mean	43.1	25.3	17.8	53.4	45.4	28.1	17.3	62.2

the over-all 50 per cent increase found at 22 hours is seen to have occurred as early as 8 hours after the introduction of anaërobiosis (Table 5). When it is recalled that gastrulation in nitrogen ceases shortly after this period (*ca.* 10 hours), the abrupt cessation of development appears to be an immediate response to the appearance of a 50 per cent rise of inorganic phosphate. The later phase of the long anaërobic period (between 10 and 22 hours), characterized by the absence of any further development, is a period of waning lactic acid production (Barth, unpublished). Anaërobic glycolysis has become too feeble to meet the triple demand of supplying energy to phosphorylation, to development, and to maintenance of the gastrula. Only the latter task is now accomplished.

TABLE 5

CHANGES IN THE DISTRIBUTION OF PHOSPHATE FRACTIONS IN THE GASTRULA DURING 2–16 HOURS' ANAËROBIOSIS

Absolute values, expressed in micrograms per 100 eggs, are mean values from 5–10 duplicate analyses.

Hours in Nitrogen	Phosphate					Significance of Differences Expressed as Inorganic/Total × 100			
	Total	Inorganic	Labile	Per Cent (Inorganic/Total)	Per Cent Increase of Inorganic	Hours Nitrogen	Mean	t	P
0	44.9	21.4	23.5	47.8		0	48.0	4.24	<0.01
						2 and 4	56.9		
2	47.8	26.3	21.6	55.0	14.1				
						0	48.0	4.95	<0.01
4	46.2	26.7	19.5	57.7	24.7	8 and 16	68.1		
8	50.8	35.3	15.5	68.8	50.4	2 and 4	56.9	3.10	0.01
						8 and 16	68.1		
16	46.9	32.1	14.9	68.6	50.0				

THE EFFECT OF SODIUM AZIDE ON DEVELOPMENT AND PHOSPHORYLATION

Azide is no longer considered solely as a cyanide-equivalent inhibitor, depressing respiration through its effect on the cytochrome system. It has been suggested recently (Meyerhof, 1945) that azide interferes with the energy transfer of tissues by inhibiting specifically their ATP-ase activity.

In preliminary tests, based on the report of Spiegelman and Moog (1945), we found that gastrulation was completely arrested at concentrations of 0.004 M and 0.005 M NaN_3 in 0.1 Ringer's solution at pH 6.7. If the eggs were washed free of azide and returned to 0.1 Ringer's within 30 minutes, development was resumed. Further exposure to azide resulted in irreversible inhibition of development and finally in cytolysis.

The results of phosphate analyses on eggs exposed to azide for 30 minutes are contrasted (Table 6) with the content in "total and inorganic" phosphate of eggs kept in 0.1 Ringer's for the same period. Azide is seen to inhibit development at the expense of a 35 per cent increase of inorganic phosphate arising from breakdown of ATP. Azide, possibly by inhibiting aërobic respiration, may deplete the energy available for the phosphorylation of ATP.

COMPARISON OF HYBRID AND NORMAL GASTRULAE IN THE ANAËROBIC BREAKDOWN OF LABILE PHOSPHATE

In a study of the respiration and lactic acid production of hybrid and normal gastrulae, Barth (1946) found that both energy sources are stabilized at the early gastrular level in the hybrid eggs in which gastrulation fails to occur. Since these "arrested gastrulae" live for 4–5 days at 18° C. with no further development, they are available for examination of the locus of the metabolic block. Since both aërobic and anaërobic glycolysis showed similar inhibition, Barth suggested that an energy *coupling* mechanism of the hybrid eggs was disturbed. Such a coupling mechanism would be found in the phosphate transfer system, participating as it does in both respiration and glycolysis. The breakdown of ATP and the production of inorganic phosphate during 10- and 22-hour anaërobiosis were therefore compared in hybrid and normal gastrulae (Table 7). All eggs were Stage 10 at the beginning of the experiment. Hybrid gastrulae remained at Stage 10 for 10 and 22 hours in both air and nitrogen. Normal aërobic gastrulae had developed to Stage 11 at 10 hours and Stage 12 at 22 hours. Normal anaërobic gastrulae, which reached early Stage 11 at 10 hours, had not developed further at 22 hours. Since the values for 10- and 22-hour breakdown were essentially the same, they were averaged together for presentation in Table 7. "Recovery" was established when tested for in one experiment on hybrid gastrulae.

It is apparent from the data in Column I that (1) there is little difference between hybrid and normal gastrulae in the quantity of ATP and inorganic phosphate at early gastrulation and (2) the hybrid gastrulae, like the normals, show an anaërobic increase of inorganic phosphate and decrease of labile phosphate. Columns II and III of Table 7 reveal, however, that the hybrid gastrulae exceed the normals in the anaërobic breakdown of labile phosphate and in the increase of inorganic and total phosphates. The percentage of change during anaërobiosis in normal gastrulae is 20–30 per cent that of the hybrids for all phosphate fractions studied. In Columns IV and V of Table 7 it is seen that, in terms of absolute values, the excessive release of inorganic phosphate in nitrogen which characterizes the hybrids may be accounted for, in the main, by their greater increase in total phosphate. The difference between hybrid and normal anaërobic breakdown of labile phosphate is negligible. It may be concluded, therefore, that some phosphate ester is more sensitive in hybrid gastrulae than in

TABLE 6

THE EFFECT OF 0.005 M NaN_3 AT pH 6.7 ON PHOSPHORYLATION IN THE GASTRULA

Each phosphate value, expressed in micrograms per 100 eggs, represents duplicate analyses.

Treatment	Phosphate			Inorganic/Total × 100	Per Cent Increase in Inorganic
	Total	Inorganic	Labile		
30 minutes in 0.1 Ringer's	50.3	22.2	28.1	44.4	34.7
30 minutes in NaN_3	49.0	29.9	19.1	61.4	.....

normal to the effects of lack of oxygen. Since anaërobic lactic acid production (source of phosphorylative energy) is 25 per cent lower in hybrids than in normals (Barth, 1946), a lowered capacity to keep metabolic intermediaries in the phosphorylated condition might be expected in hybrid gastrulae.

DISCUSSION

Phosphorylation of intermediary metabolites provides an energy-accumulating mechanism that may be linked to a wide variety of endergonic biological processes (as in muscle, kidney, etc.). The present work indicates that such phosphate-bond energy is applied to the early developmental systems of the amphibian eggs.

The small amount (cf. for muscle, Kalckar, 1942) of labile phosphate demonstrable during early amphiban development does not vary with stage of development but appears, rather, to be dependent upon the quantity of energy available from either respiration or glycolysis.

That these exergonic reactions may, however, become limiting for the processes of development is indicated by the differential response to anaërobiosis at various stages of development. The lowered energy demands of cleavage stages in the amphibian egg are met adequately by the energy bound in phosphate esters as a result of anaërobic glycolysis. The labile phosphate level of eggs during cleavage was affected but slightly by the imposition of the anaërobic condition. Development, i.e., cleavage or mitosis, proceeded at the normal aërobic rate.

The higher Q_{O_2} characteristic of gastrulation (and neurulation), however, reflects demands upon the energy-yielding systems that are in excess of the supply available from anaërobic glycolysis. It would appear that the energy bound in labile phosphate compounds can support gastrulation for a time (to Stage 11) but that thereafter insufficient glycolytic energy is available for the multiple transfer to development, maintenance, and phosphorylation mechanisms. This

TABLE 7

COMPARISON OF HYBRID AND NORMAL GASTRULAE FOR CHANGES IN PHOSPHATE FRACTIONS DURING ANAËROBIOSIS

(Average of 6 Experiments Run in Duplicate)

Phosphate Fraction	(I) Micrograms per 100 Eggs		(II) Per Cent Change in Nitrogen	(III) Anaërobic (N/H×100) Difference between H and N	(IV) Change during Anaërobiosis in Micrograms per 100 Eggs	(V) Difference between H and N in Micrograms per 100 Eggs
	Air	Nitrogen				
Total	N,* 43.0 H,† 38.7	48.8 46.1	+13.5 +19.2	70.3	5.8 7.4	1.6
Inorganic	N, 19.3 H, 17.3	29.9 30.2	+55.0 +74.6	73.7	10.6 12.9	2.3
Labile	N, 23.7 H, 21.4	18.9 15.9	−20.2 −25.7	78.6	4.8 5.5	0.7
Inorganic/Total×100	N, 44.8 H, 44.7	61.3 65.5	+36.8 +44.6	79.0	.....	.....

* N = Normal eggs. † H = Hybrid eggs.

effect, though not tested for in the present experiments, should be even more striking at neurulation, a stage characterized by an even higher oxygen consumption.

The phenomenon of "overshoot" in respiration in gastrulae, to which air is readmitted after a period of oxygen deprivation, gives rise to some interesting possibilities. Brachet (1934) found that such eggs exceeded normal aërobic gastrulae at the same stage in their rate of respiration. The "overrecovery" of labile phosphate content described above is not, therefore, unexpected. "Overshoot" in Q_{O_2} provides sufficient energy not only for renewed development, but for resynthesis of the depleted energy-rich phosphate compounds necessary for maintenance and development. It may be noted here, since anaërobiosis is used above as a tool, that the reversibility of its effect on phosphorylation provides adequate proof that the appearance of free inorganic phosphate at the expense of breakdown of labile phosphate is not simply an artefact of cytolysis but the reflection of a compensatory and reversible metabolic reaction.

The quantitative variability in labile phosphate response to anaërobiosis (cf. Table 3 breakdown with Table 3) is emphasized by the thus far erratic participation of a noncalcium-precipitable ester in anaërobic metabolism. In some experiments only $\Delta 7$-minute phosphate decreased under anaërobic conditions. At other times, however, an extrinsic ester, whose nature remains obscure, was brought into play. Phosphocreatine, a possible participant in the phosphorylative scheme, has not yet been investigated in the amphibian egg (except for the technically questionable experiments of Zielinski [1937]).

That the equilibrium between phosphorylated intermediaries of respiration and glycolysis is likely to be sensitive has been demonstrated adequately by experiments on muscle. Until we have been able to establish the conditions that determine the equilibrium between the various components of the phosphate-bond transfer system of the amphibian embryo, an approach more fruitful than simple quantitative determination of phosphorylated intermediaries would appear to be an attack upon the *rate* of turnover of phosphate-bond energy under various experimental conditions (i.e., ATP-ase activity).

The comparison of hybrid ("arrested") and normal gastrulae here may provide a guide. Although the absolute breakdown of labile phosphate was about the same in hybrid and in normal gastrulae, the former drew upon an additional ester during the same anaërobic period. Were the ATP-ase activity lower in the hybrids, an extrinsic ester not normally drawn upon might now be attacked. An alternative explanation is equally possible. The extrinsic ester may be one directly concerned in the transfer of energy to maintenance and development, its state of phosphorylation dependent upon energy donated by ATP. When the reaction $ADP+P \rightleftharpoons ATP$ is pushed to the left during anaërobiosis, insufficient energy is available to phosphorylate the unknown ester.

SUMMARY

The present work attacks the problem of whether phosphate-bond energy plays a role in the transfer of metabolic energy to developmental processes in the amphibian egg. ATP was found to be present in approximately the same amounts from early cleavage through early neurula. The introduction of anaërobic conditions resulted in a decrease of ATP, with corresponding increase of

inorganic phosphate at all stages investigated.

The relative amount of ATP breakdown at different stages in response to nitrogen was found to parallel the amount of inhibition of development. Cleavage proceeds normally in nitrogen with relatively little ATP breakdown, while gastrulation is arrested in response to a nitrogen atmosphere, with correspondingly greater ATP breakdown. The release of inorganic phosphate from ATP appears to occur rapidly after exclusion of air and to be readily reversible. Sodium azide inhibition of gastrulation was found to be accompanied by increased inorganic phosphate at the expense of the ATP content of gastrulae.

Evidence that phosphate-bond energy is coupled to gastrulation came also from the study of arrested hybrid gastrulae, whose lower rate of respiration and glycolysis was found to be accompanied by a decreased capacity to keep ATP (as well as an unidentified organic phosphate ester) in the phosphorylated condition.

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RIBOFLAVIN AND OTHER FLUORESCENT COMPOUNDS IN A DEVELOPING EGG (ORTHOPTERA)[1]

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THAT most living biological materials contain riboflavin seems well established. Its role in the general nutritional requirements and growth of organisms has been repeatedly pointed out and confirmed. Some ideas as to its fundamental mode of action in the living cell have been stressed, and, in particular, attention has been given to the part played by it in conjunction with certain enzyme activities. Warburg and others (see Rosenberg, 1942, for complete bibliography), for example, have definitely connected it with cellular oxidative

[1] Aided by a grant from the Rockefeller Foundation for work on the physiology of the normal cell.

and respiratory functions. Its chemical constitution and much of its purely physicochemical properties have also been rather extensively studied. Its fluorescent nature, which is constant under controlled conditions, makes possible its quantitative determination. The present paper is concerned with the results of a study of the riboflavin content and the changes in the fluorescence of the egg of the common grasshopper, *Melanoplus differentialis*, during the entire course of its development.

METHOD

Eggs of the grasshopper (*M. differentialis*), of known ages and history, were obtained by methods previously described (Slifer, 1932). Eggs from several pods of the same age were thoroughly mixed, and appropriate numbers were then selected in order to insure random samples for fluorescence determinations. Checks, using eggs from the same pod, were also carried out. One hundred eggs were carefully selected, washed in distilled water and ground in a mortar in 30 cc. of 0.25 N sulphuric acid. After standing for a short period, the acid extract was filtered, and to the clear filtrate were added 5 cc. 2.5 N sodium acetate and distilled water to 100 cc. total volume. Acid extraction of eggs was also carried out at higher temperatures (up to 100° C.), as indicated below. Lipids were removed from the egg extracts by high-speed centrifugation, but, since no differences in results were noted, such procedures were deemed unnecessary. All filtrations were carried out in light-free chambers, and extracts were allowed to remain exposed to the light of the room for the absolute minimum of time.

Sulphuric acid (0.25 N) was found to be most satisfactory in extracting riboflavin and other water-soluble fluorescent compounds from the eggs. Other reagents, such as acetone and methanol, were employed but were discarded because of the fact that they themselves fluoresced and gave no more consistent results than those for sulphuric acid. In order to separate riboflavin from other heat-labile fluorescing compounds, extracts were heated at 100° C. for various periods, but with no appreciable differences in results from the nonheated extracts.

The pH of the extracts was adjusted to 4.5 by the addition of sodium acetate and to 10–11 by potassium hydroxide.

Riboflavin determinations were carried out according to the general method described by Hodson and Norris (1939), an indirect method which makes use of the fluorophotometer. The fluorophotometer, Model B, of Pfaltz and Bauer, was employed throughout. Briefly, the procedure was as follows: For the exciting light source, an 85-watt mercury capillary arc at 110 volts, A.C., was employed. Filter combinations with a peak between 4300 A and 4400 A were used to produce a pure blue activating light. The fluorometric photocell was protected by an orange filter, which cut out all wave lengths lower than 5200 A. Stock riboflavin solution (20 γ per cubic centimeter) was made up by dissolving 20.0 mg. of riboflavin (Merck's) in doubly distilled water with the addition of a few drops of glacial acetic acid and water up to 1,000 cc. This solution was stored in an amber-colored bottle in the dark at 5° C. A standard riboflavin solution (0.1 γ per cubic centimeter) was made up daily by diluting 1 cc. of the stock solution to 200 cc. with distilled water. Appropriate amounts of sodium acetate were added to the standard solution to compare with those added to the egg extracts. The fluorophotometer was checked daily with the freshly prepared standard solution (0.1 γ per cubic centimeter) before

determinations. Readings on the scale of the multiple-mirror galvanometer were adjusted so that, with the standard solution in the optical cell, a scale value of 35 was obtained. From 15 to 20 cc. of the solutions to be tested were used in the cuvettes. Calculations and procedures were as follows: The optical cell containing the unknown was placed in the fluorophotometer, and a reading (F) was made. The standard solution of riboflavin was next placed in the optical cell, and a reading (R) was made on it. After

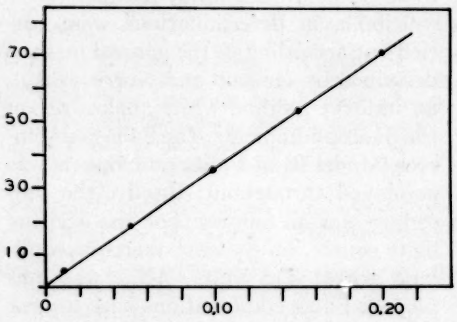


FIG. 1.—Calibration curve for fluorophotometer. *Ordinate*, scale reading of galvanometer; *abscissa*, concentration of riboflavin in gammas per cubic centimeter. Shows linear relationship between concentration and scale readings.

each reading of the fluorophotometer a few crystals of sodium hydrosulphite were added to the contents of the optical cell to reduce the riboflavin, and another reading (B) was made. Then $(F - B)/(R - B) \cdot rz$ = riboflavin per cubic centimeter in unknown (r = concentration of riboflavin in gammas per cubic centimeter of standard solution; z = dilution factor) (see Fig. 1 for calibration curve).

Riboflavin in the egg extracts, reduced by the addition of sodium hydrosulphite and then reoxidized by shaking with air, was recovered quantitatively. Substances interfering with riboflavin determinations were destroyed or at least irreversibly affected by treating extracts with potassium permanganate and later with hydrogen peroxide. Nonreversible reducible compounds are in this way kept out of the field of influence while the riboflavin is being determined. Both the above methods for reducing the fluorescent compounds were employed in the present experiments.

In any indirect method of riboflavin determination caution must be used in seeing that no interfering compounds are present in the extract under examination. Additions of known amounts of riboflavin to such extracts must be recovered quantitatively if results found are to be valid. Many such control experiments have been carried out and will be indicated in what follows.

Fluorescence of riboflavin is dependent to a marked degree upon the pH of the surrounding medium. Values of pH between 4 and 6 give practically 100 per cent fluorescence (Scott *et al.*, 1946). In highly alkaline solution—pH 10–11—riboflavin loses its fluorescence (Kahler and Davis, 1940). Determinations of riboflavin in the present experiment were carried out at a pH of 4.5. Practical use of the effects of strong alkaline solutions was made in separating riboflavin from other fluorescing substances.

Separations of the diffusible and nondiffusible fluorescent compounds in the egg extracts were carried out by dialysis with Visking Cellophane sausage casing at 0° C. for periods of 24 or more hours.

Chromatographic analyses have been employed with whole extracts as well as with dialized portions and will be described in detail at a later date.

All results are expressed in terms of gammas of riboflavin per egg unless otherwise indicated.

RESULTS

Since the egg of the grasshopper is a cleidoic one, little or none of the organic

materials essential for its development are supplied from its external environment. Water and gases (oxygen and carbon dioxide) are perhaps the only substances which pass in and out of the egg during its entire development. Whatever the egg's complement in fluorescent materials, they must be derived from maternal sources or are themselves manufactured within the egg. Such a system, therefore, seems well adapted to a quantitative analysis of the problem at hand.[2]

diet, health, etc., of the females may well account for differences noted. Mean or average figures are quite constant for eggs of similar ages when kept at 25° C., as noted below.

Results for large numbers of fluorometric determinations are graphically depicted in Figure 2, while in Table 1 are given data as obtained from different lots of eggs and nymphs. An examination of these shows that, throughout the egg's development, *the total fluorescence at pH 4.5* remains practically constant. The same general picture holds for the nymphs when newly hatched and unfed. Such results suggest that the original fluorescent materials remain constant in amount in the egg and that, even though new fluorescent compounds are formed, their fluorescent properties may well be derived from the original riboflavin present. Data supporting such a view are given in what follows.

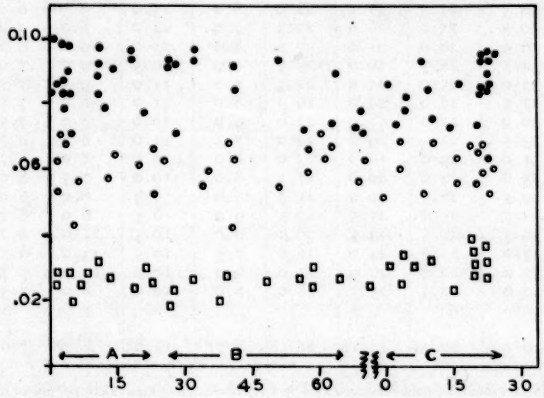


FIG. 2.—Shows fluorescence of egg extracts at various periods of development. pH = 4.5 *Ordinate*, gammas riboflavin (or equivalent) per egg; *abscissa*, age in days. (*A* = prediapause, *B* = diapause, *C* = postdiapause.) *Solid dots*, total fluorescence; *open circles*, riboflavin; *squares*, nonreducible fluorescent material.

TOTAL FLUORESCENCE OF EGG EXTRACTS AT pH 4.5

Analyses have been carried out on eggs all from one pod, and hence from the same female, as well as on different lots of eggs of the same developmental ages but coming from assorted pods. Variations in results are of equal magnitude, whatever the procedure. Such factors as

Dissection and separation of embryos from the yolk show that the fluorescent compounds are originally in the yolk and pass to the embryo as development and utilization of the yolk proceed (Table 2).

[2] The part played by riboflavin in the development of the normal embryonic cell seems of particular interest in view of the many observations concerning its intimate relationships in connection with the growth of various types of tumors in the fully formed organism (see recent article by A. C. Griffin and C. A. Baumann, The effect of certain azo dyes upon the storage of riboflavin in the liver, Arch. Biochem., 11:467–76, 1946).

TABLE 1*

FLUORESCENCE AND REDUCIBILITY OF EXTRACTS OF EGGS AT DIFFERENT PERIODS OF DEVELOPMENT AS WELL AS OF NEWLY HATCHED NYMPHS

Total Fluorescence (pH 4.5)			Reducible (pH 4.5)			Nonreducible (pH 4.5)			Total Fluorescence and Nonreducible (pH 10–11)		
Prediapause and diapause	Postdiapause	Nymph	Prediapause and diapause	Postdiapause	Nymph	Prediapause and diapause	Postdiapause	Nymph	Prediapause and diapause	Postdiapause	Nymph
24.0	40.0	25.0	16.5	25.5	18.0	7.5	14.5	7.0	7.7	33.0	27.0
25.0	36.5	28.5	18.0	25.5	19.5	7.0	11.0	9.0	7.0	15.2	42.0
29.0	34.0	29.0	21.0	21.0	21.0	8.0	13.0	8.0	8.2	26.0	30.7
29.5	42.5	30.5	21.5	27.5	22.0	8.0	15.0	8.5	6.2	37.0	42.0
28.0	40.0	30.5	21.0	26.0	22.5	7.0	14.0	8.0	7.2	24.3	34.0
34.0	33.0	36.0	26.0	21.0	27.0	8.0	12.0	9.0	9.0	34.0	37.2
29.5	30.5	34.5	22.5	20.0	25.5	7.0	10.5	9.0	7.0	42.0	46.0
32.0	33.0	32.0	25.0	22.0	22.5	7.0	11.0	9.5	7.2	33.0	45.5
26.0	37.0	27.5	18.0	26.0	19.5	8.0	11.0	8.0	7.5	28.0	53.0
31.0	32.0	30.0	22.0	22.0	22.0	9.0	10.0	8.0	7.5	27.0	33.7
28.5	40.0	33.5	21.0	29.0	24.0	7.5	11.0	9.5	9.7	21.0	28.7
28.0	24.0	31.0	20.0	16.0	23.0	8.0	8.0	8.0	7.7	30.7	29.0
27.0	30.0	25.0	19.0	20.0	17.5	8.0	10.0	7.5	8.0	21.5	34.5
29.0	28.5	29.5	21.0	20.0	21.5	8.0	8.5	8.0	6.0	21.0	33.0
30.0	31.0	32.5	21.0	21.5	17.5	9.0	9.5	6.0	8.7	25.5	31.0
29.5	34.0	30.5	21.5	23.5	20.0	8.0	10.5	11.0	8.2	22.6	35.0
24.5	34.5	35.0	17.0	24.0	21.0	7.5	10.5	13.0	6.5	21.3	32.0
18.5	38.0	32.0	20.0	26.0	21.0	6.0	10.0	12.0	7.7	30.3	33.0
28.0	33.0	30.0	12.5	25.5	18.0	8.0	10.0	8.0	7.5	33.0	34.5
Av. 27.9	34.2	30.1	20.24	23.26	21.18	7.71	11.05	8.78	7.61	27.70	35.88

* All values expressed in galvanometer-scale readings; 33.5 = 0.1 γ riboflavin or equivalent per egg or nymph.

TABLE 2*

GAMMAS OF RIBOFLAVIN OR ITS REDUCIBLE DERIVATIVES IN WHOLE EGGS, YOLK, AND AND EMBRYOS FROM EGGS

Egg	Yolk	Embryo
0.0636	0.0485	0.0151
0.0552	0.0403	0.0149
0.0706	0.0587	0.0119
0.0733	0.0565	0.0168
0.0947	0.0758	0.0189
0.0657	0.0418	0.0239
0.0805	0.0358	0.0447
0.0805	0.0282	0.0523
0.0805	0.0387	0.0418

* Series arranged from early to late postdiapause stages.

TOTAL FLUORESCENCE OF EGG EXTRACTS AT pH 10–11

Total fluorescence of egg extracts is doubtless produced by a variety of chemical compounds and is in itself a poor criterion of the presence of any specific types of substances other than those with fluorescent properties. A beginning at a separation and analysis can be made by using some of the well-known and rather specific properties of riboflavin, viz., its inability to fluoresce in an alkaline medium (pH 10–11). Such procedure has been followed, and the results of experiments are shown graphically (Fig. 3) as well as in tabular form (Table 1). An inspection of these data shows quite clearly that, as development proceeds, the amount of fluorescence at pH 10–11 (and not due to riboflavin as such) increases during postdiapause to a maximum in the newly hatched nymph.

Freshly laid eggs contain an alkaline fluorescent fraction which is not ribo-

flavin (Fig. 4; Table 1). The amount of it is amazingly constant throughout development and is usually found to make up less than one-third of the total fluorescence of the egg. As a matter of fact, this fraction, as will be shown below, is quite distinct from others with similar fluorescent properties but is formed later in the egg's development. That it is present in the egg from the start is certain. During the development of the egg, especially in postdiapause, the amount of alkaline fluorescing materials shows a marked increase, which continues during subsequent development and in the nymph reaches a value practically equal to that for the total fluorescence of the egg at pH 4.5 (Figs. 3, 4; Table 1). Obviously, transformation of the original fluorescent materials (riboflavin) must in some way be related to, or connected with, these changes. Further analyses, as shown below, give supporting evidence for such a view.

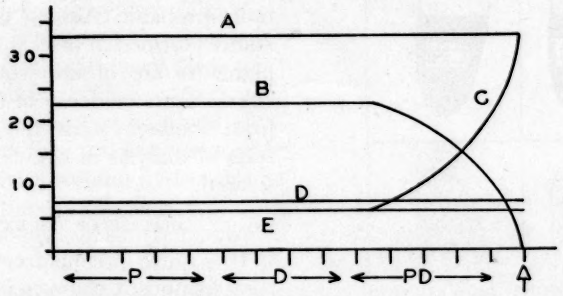


FIG. 3.—Shows amounts of fluorescent materials in egg and nymph. *Ordinate*, scale reading of galvanometer (33.5 = 0.1 γ riboflavin per egg); *abscissa*, time in days (5-day periods indicated); *P* = prediapause; *D* = diapause; *PD* = postdiapause; *arrow* = newly hatched nymph. Curve *A* = total fluorescence at pH 4.5. Curve *B* = riboflavin. Curve *C* = fluorescent material formed from riboflavin (fluoresces at pH 4.5 and pH 10; is diffusible; reducible with sodium hydrosulphite at pH 4.5). Curve *D* = nonreducible, fluorescent fraction at pH 4.5, inherent in egg from laying; is nondiffusible. Curve *E* = nonreducible, fluorescent fraction at pH 10, inherent in egg from laying; is nondiffusible.

REDUCIBLE AND NONREDUCIBLE FLUORESCENT COMPOUNDS

As previously pointed out, one of the outstanding characteristics of riboflavin is its marked ability to be reduced by such compounds as sodium hydrosulphite and its reversibility upon shaking with air. Total fluorescence of egg extracts at pH 4.5 is due to riboflavin plus other substances which fluoresce in acid medium. By treating such extracts with sodium hydrosulphite, riboflavin is quickly reduced and then becomes nonfluorescent; the remaining fluorescence is therefore due to nonreducible compounds other than riboflavin.

An inspection of Figures 2, 3, and 4 and Table 1 shows that the riboflavin content of the egg is fairly constant and remains so until very late in the egg's development (postdiapause) (Fig. 3). No significant changes in its concentration during the active phases of the egg's development, as judged by fluorescence and reducibility, are noted. As the embryo grows in size, increasing amounts of riboflavin or its derivatives can be shown to leave the yolk and enter the embryo (Table 2).

The nonreducible fraction, fluorescing at pH 4.5, approximates about one-third of the total fluorescence of the egg ex-

tract and remains so throughout development (Table 2; Fig. 1).

When sodium hydrosulphite is added to egg extracts in an alkaline medium (pH 10–11), no reducible compounds are found, suggesting the absence of riboflavin and the presence of other irreducible compounds (Table 1). Included in these nonreducible fluorescing substances are those present in the egg from the beginning, as well as others apparently formed in increasing amounts during the development of the embryo (Fig. 3).

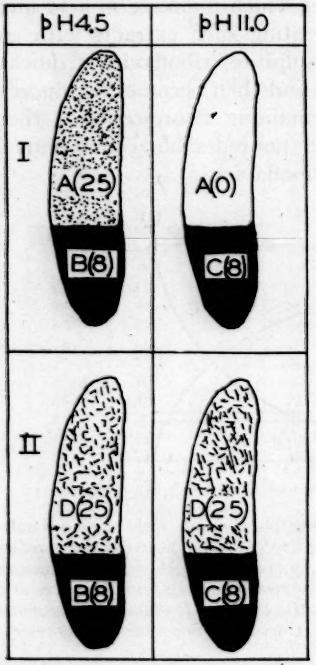


FIG. 4.—A schematic semi-quantitative sketch of eggs at pH 4.5 and pH 11 to show general relations and changes in fluorescence of egg at different periods in its development. *I*, for prediapause and diapause eggs; *II*, for late postdiapause eggs. *A* = riboflavin; *B* = inherent, nondiffusible, fluorescing fraction (pH 4.5); *C* = inherent, nondiffusible, fluorescing fraction (pH 11); *D* = fluorescent fraction formed from riboflavin, flouresces at pH 4.5 and 11; is diffusible and reducible with sodium hydrosulphite at pH 4.5. Figures in brackets represent scale readings ($\frac{1}{3}$3.5 = 0.1 γ riboflavin per egg) and thus show relative amounts of various compounds in egg.

The possible functions of riboflavin and other fluorescent compounds during the egg's development become questions of considerable interest in view of the above results. Since the total riboflavin remains constant for such a long period during development, it would seem to indicate either that it is not used or that it possibly becomes loosely incorporated into other fluorescent compounds. The latter of these suggestions appears the more probable. Among fluorescent substances formed in insects, the pterines or pigments are of importance (Hopkins, 1941). Some evidence of their formation from riboflavin seems indicated when results of dialysis of egg extracts are considered.

DIALYSIS OF EGG EXTRACTS

It is quite obvious from the foregoing that significant changes in the nature of the fluorescent substances of the egg take place during its course of development and especially during late postdiapause. For possible separation and isolation of these fluorescing fractions, dialysis of egg extracts was carried out. Extracts were dialyzed against 0.25 N sulphuric acid, as well as 0.25 N sulphuric acid plus sodium acetate, and no significant differences in results in the two types of procedures were noted. Rather detailed quantitative check experiments were run with pure riboflavin, as well as with extracts plus riboflavin, and results were found to agree extremely well (Table 3).

Several points of interest are obvious from the results of dialysis of egg extracts. First, riboflavin, as found in egg extracts as well as when added in a pure state, is readily diffusible and can be recovered quantitatively in the system after periods of 24 or more hours of dialysis (Table 4). Second, the inherent fluo-

rescent materials (other than riboflavin) present in the egg at the beginning of development and making up the inherent acid and alkaline nonreducing fluorescent compounds (see above) are nondiffusible and remain practically constant in amount within the dialyzing shell (Fig. 5). Third, the alkaline and acid fluorescing compounds (exclusive of the above), formed especially during the latter part of the egg's development, are readily diffusible and as such are recovered in both the outer and the inner solutions of the dialyzing system (Table 4). With frequent changes of the outer solutions, complete separations of these compounds are produced (Figs. 5, 6, 7). Not only do they fluoresce in acid and alkaline media, but they are also reduced in acid medium with sodium hydrosulphite (Fig. 4), as is pure riboflavin.

Since the amounts of substances which fluoresce in both acid and alkaline media increase in postdiapause to a value approximately equal to the original total fluorescence of the egg, it becomes of some interest to seek a possible explanation of such phenomena. During the development of an insect, pterines or pigments are known to appear (Hopkins, 1941). Concerning the details of such formations, little is apparently known. From the results of the present studies of the fluorescent compounds produced during the course of development in the grasshopper egg, we are led to consider them to be largely pterines. As a class of compounds, they show some characteristic fluorescent properties similar to those described for pterines (Becker, 1936). Their exact chemical origins are at present poorly understood, and any suggestions are based on purely empirical grounds. Pterines are thought by some

TABLE 3

SHOWS AMOUNT (IN γ) OF PURE RIBOFLAVIN ADDED TO INSIDE OF DIALYZING SHELL AND AMOUNTS RECOVERED IN SYSTEM AFTER 48 HOURS OF DIALYSIS AGAINST 0.25 N H_2SO_4

Amount Added (before Dialysis)	Amount Recovered
3.00	3.09
3.00	2.88
6.00	6.27
3.00	2.78
3.00	3.22
3.00	3.36
3.00	2.86
6.00	5.52
3.00	2.98

TABLE 4*

RESULTS OF TYPICAL DIALYZING EXPERIMENTS WITH EXTRACTS OF EGGS AND NYMPHS

Stage	After Dialysis			Total Fluorescence	
	Original Extract	Inside of Shell	Outside of Shell	Before Dialysis	After Dialysis
Prediapause	0.0636	0.0288	0.0393	6.36	6.81
Diapause	0.0537	0.0209	0.0358	5.37	5.67
Postdiapause	0.0772	0.0303	0.0273	2.31	2.54
Postdiapause	0.0865	0.0409	0.0455	8.65	8.64
Postdiapause	0.0642	0.0209	0.0418	6.42	6.27
Postdiapause	0.0545	0.0212	0.0339	5.45	5.46
Postdiapause	0.0561	0.0224	0.0149	1.68	1.57
Nymph	0.0403	0.0153	0.0153	1.21	1.38
Nymph	0.0666	0.0358	0.0433	6.66	7.90
Nymph	0.0403	0.0153	0.0153	1.21	1.38

* Figures expressed in gammas of riboflavin per cubic centimeter except where total amounts are indicated.

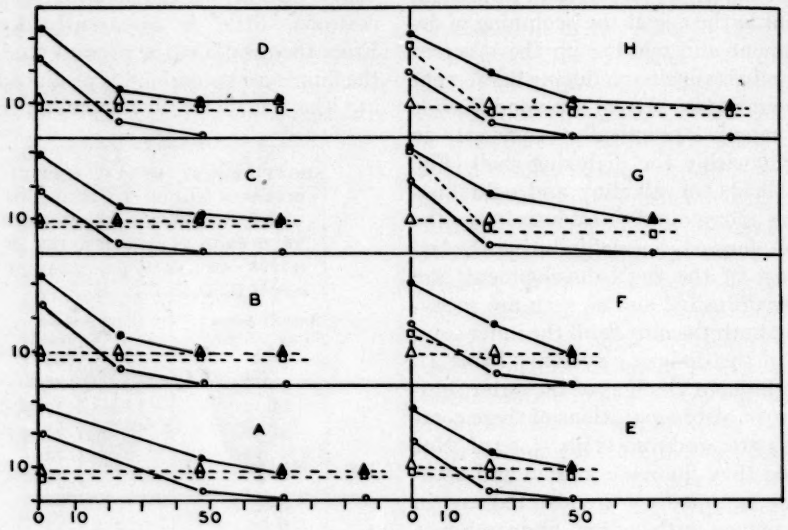


FIG. 5.—Shows results of dialysis of egg extracts of different ages against 0.25 NH_2SO_4. *Ordinate*, scale reading (33.5 = 0.1 γ riboflavin per egg); *abscissa*, time of dialysis in hours (outside solution renewed every 24 hours); *solid dots*, total fluorescence at pH 4.5; *open circles*, riboflavin or compounds reducible by sodium hydrosulphite at pH 4.5; *triangles*, nondiffusible, nonreducible friction, fluorescing at pH 4.5; *squares*, nondiffusible fraction fluorescing at pH 11 *plus* diffusible substances formed from riboflavin and fluorescing at pH 11. *A* and *B* = prediapause eggs, *C* and *D* = diapause eggs, *E* = early postdiapause eggs, *F* = middle postdiapause eggs, *G* and *H* = late postdiapause eggs.

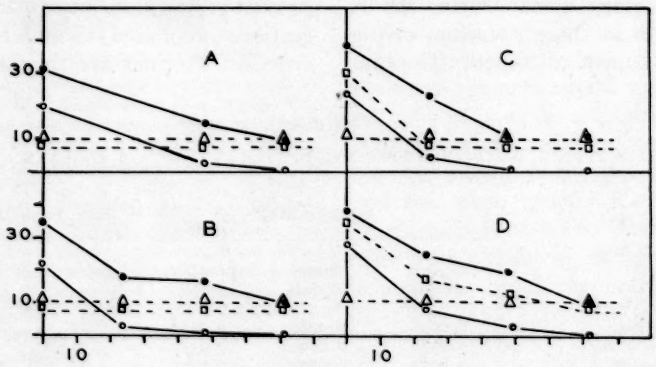


FIG. 6.—Shows results of dialysis of a single lot of eggs during postdiapause development (otherwise as in Fig. 5). *A* and *B* = early postdiapause; *C* and *D* = late postdiapause.

to be derived from uric acid or related substances (Hopkins, 1941). In view of the results of the present study, it is conceivable that they may well be made up, in part at least, from the riboflavin molecules plus other organic compounds in the egg. Their fluorescence is probably conditioned by their riboflavin complement, and this, in turn, must be loosely combined with other types of organic radicals. The total reducible fluorescence of the egg extract at the time of hatching, as well as that of the newly hatched nymph, is approximately equal to, or at least approaches, that for the original riboflavin concentration (see Fig. 3), suggesting a marked economy in the organism's use of such compounds. Some ideas as to the possible intimate chemical changes from riboflavin to a pterine are indicated in diagram on following page.

Some light on the question of whether riboflavin, as such, first passes to the embryo to be transformed into other fluorescing compounds or is made over in the yolk and then transferred can be gained by examination of results of dialysis experiments on whole eggs and embryos minus yolk (Figs. 5, 6, 7). From an examination of these, one sees that diapause or early postdiapause eggs show little change in free riboflavin. Embryos from these eggs also show fluorescent compounds in relatively the same general proportions as in the entire egg (yolk *plus* embryo). In late postdiapause (Figs. 5, 7) both embryos and eggs still show the same relative composition of fluorescent

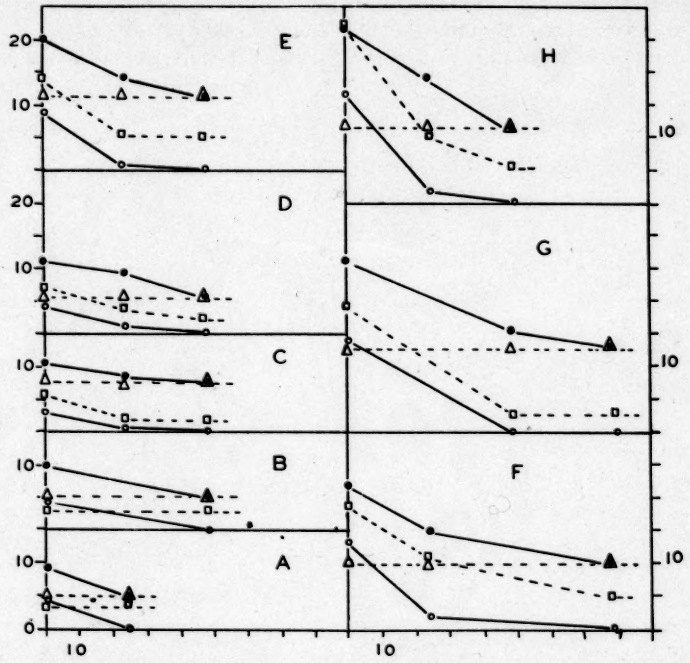


FIG. 7.—Shows results of dialysis if embryo minus yolk from eggs of Figure 6 (otherwise as in Fig. 5). *A* and *B* = early postdiapause; *C* and *D* = middle postdiapause; *E*, *F*, *G*, and *H* = successive periods in late postdiapause.

compounds, suggesting that changes in the riboflavin take place in the yolk as well as in the embryo proper. Study is being given this point to learn further as to the exact time and types of compounds produced, and the results of these investigations will be reported at a later date.

Rates of formation of the alkaline, nonreducible fluorescent compounds in the embryo minus yolk, as well as in the entire egg, follow definite autocatylitic curves, as indicated in Figure 8.

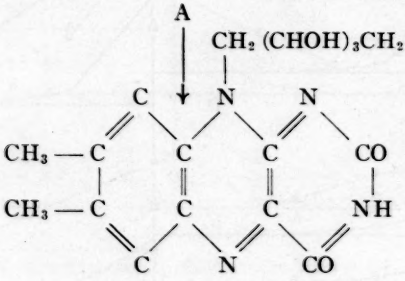
SPECTRAL TRANSMITTANCE

Studies of the spectral transmittance of egg extracts with a Coleman spectrophotometer have been carried out and will be reported in detail at a future date. Suffice it to add at this time that marked changes in the spectral properties of the egg extract occur and that these, in turn, corroborate those indicated above for changes in the fluorescent compounds produced (Fig. 9).

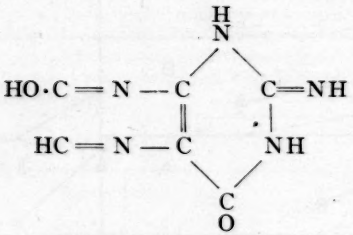
DISCUSSION

Investigations on the presence of riboflavin and related fluorescing compounds in insects are indeed few, but among the more recent ones those of Busnel and Drilhon (1942), Metcalf and Patton (1942), and Metcalf (1943) are of considerable interest. These authors show the presence of riboflavin in most insects and in particular in the Malpighian tubules, tissues, and eggs. Busnel and Drilhon have considered at some length the origin and functions of the compound in the general metabolic states of these organisms. They clearly point out its presence in the eggs of certain insects and show a curiously marked increase in the compound as development proceeds. Details as to exact methods followed in preparing their egg extracts are lacking, and thus it is quite impossible to compare their results with those reported here. However, judging from present results, increase in the egg's fluorescence as re-

(Riboflavin)



(Xanthopterin)



(A splitting at *A*, with subsequent changes, might easily produce xanthopterin as indicated in formula?)

ported by them may well be due to increases in fluorescing compounds other than riboflavin but doubtless derived from it. It is rather difficult to see how they can explain an actual increase in the amount of riboflavin in the egg if the eggs of the forms they employed function as do the grasshopper eggs. Judging from their data, however, one is led to conclude that, had some differential method for separation of the fluorescing compounds been used, much of the reported increased fluorescence would have been found due to compounds other than riboflavin but formed from it during the course of development.

Metcalf (1943) has especially studied the riboflavin in the Malpighian tubules of the cockroach and separates the materials found into (1) a free riboflavin, which is diffusible and reducible by sodium hydrosulphite, and (2) a cytoplasmic riboflavin, which is nondiffusible and nonreducible by sodium hydrosulphite. It would be of considerable interest to have further data on this cytoplasmic riboflavin and to learn further concerning its fluorescing properties, especially to compare it with the alkaline fluorescing compounds of the grasshopper egg. Further quantitative study seems necessary before such questions can be answered satisfactorily.

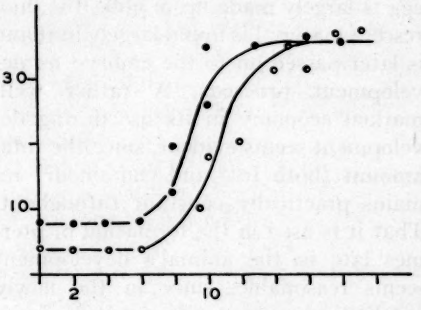


FIG. 8.—Shows increase in diffusible, alkaline (pH 11) fluorescing fraction in egg (*solid dot*) and embryo minus yolk (*open circles*) during postdiapause. *Ordinates*, scale reading (33.5 = 0.1 γ riboflavin per egg); *abscissa*, time in days indicated.

The probable functions of riboflavin in the development of the grasshopper embryo are indeed interesting when the importance of the compound for the normal well-being of other forms is considered. That adult insects apparently require and use riboflavin seems certain

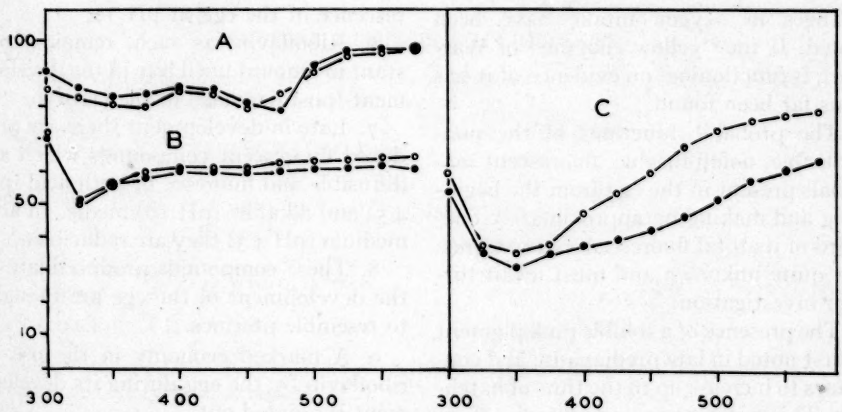


FIG. 9.—Shows spectral transmittance of riboflavin (*A*), prediapause egg extract (*B*), and of newly hatched nymphs (*C*). *Ordinate*, percentage transmittance; *abscissa*, wave lengths in millimicrons; *solid dots*, pH 4.5; *open circles*, pH 11.

from the results of Metcalf and Patton (1942). The total amount of riboflavin contained in the average grasshopper egg is approximately 0.06–0.08 γ or 15–17 γ per gram, wet weight of egg. Since the egg is largely made up of yolk, the fluorescent material is found largely in it and is later passed on to the embryo as development proceeds. A rather well-marked economy in its use during development seems evident, since the total amount (both free and combined?) remains practically constant throughout. That it is used in the formation of pterines late in the animal's development seems reasonable, since in the newly hatched nymph no free riboflavin is found. Once the animal hatches, its supply of riboflavin is obtained through the food it eats. Evidence presented by Metcalf and Patton (1942) for the adult cockroach substantiates such a conclusion.

During the development of the egg, marked changes in its oxidative exchange in the prediapause, diapause, and postdiapause stages take place, as has been repeatedly pointed out (Bodine, 1932). However, in the present experiments no changes in the riboflavin paralleling such changes in oxygen intake have been noted. If the "yellow enzyme" of Warburg is functioning, no evidence of it has thus far been found.

The probable functions of the nonreducible, nondiffusable, fluorescent materials present in the egg from the beginning and making up approximately one-third of its total fluorescence are at present quite unknown and must await further investigation.

The presence of a soluble pink pigment is first noted in late prediapause and continues to increase up to the time of hatching. This pigment is undoubtedly of an oxidation-reduction type, since after reduction it assumes a yellow color, which is readily reversible to pink. It does not fluoresce, nor does it possess any of the characteristics of riboflavin. Further study of it is being carried out, and results will be published at a later date.

SUMMARY

1. A quantitative study has been made of the riboflavin and other fluorescent compounds in the egg of the grasshopper (*M. differentialis*) throughout its entire development.

2. The average amount of riboflavin in the egg of the grasshopper is 15–17 γ per gram wet weight.

3. Total fluorescence of the egg at pH 4.5 remains practically constant throughout development.

4. The egg when laid contains riboflavin and a nonreducible, nondiffusible fraction, fluorescing at pH 4.5. This fraction makes up approximately one-third of the total fluorescence of the egg at pH 4.5.

5. A nonreducible, nondiffusible fraction, fluorescing at pH 10, is also present in the egg when laid. This fraction makes up less than one-third of the total fluorescence of the egg at pH 10.

6. Riboflavin, as such, remains constant in amount until late in the development (postdiapause) of the embryo.

7. Late in development there are produced fluorescent compounds which are diffusible and fluoresce in both acid (pH 4.5) and alkaline (pH 10) media. In acid medium (pH 4.5) they are reducible.

8. These compounds produced late in the development of the egg are thought to resemble pterines.

9. A marked economy in the use of riboflavin by the egg during its development is pointed out.

10. No free riboflavin is found in the nymph when hatched.

CHECK ON METHOD AND RECOVERY OF ADDED KNOWN AMOUNTS OF PURE RIBOFLAVIN

a) 200 eggs, postdiapause (12 days). Standard = 33.5 = 0.1 riboflavin/cc. Ground in mortar in 59 cc. 0.25 N H_2SO_4 + 1 cc. stock riboflavin (0.1 γ/cc.); filtered; added 10 cc. 2.5 N NaAc + H_2O to 200 cc. total volume.

extract = 55.0
+ crys. = 11.0 } 44.0 (0.1314) γ/cc.

extract + KOH = 8.0
+ crys. = 10.0

Extract dialyzed 24 hours versus 0.25 N H_2SO_4 + NaAcc (pH 4.5)

Inside of shell (*30 cc. vol.*):

extract = 27.0
+ crys. = 13.0 } 14.0 (0.0418) γ/cc.

extract + KOH = 8.5
+ crys. = 9.5

Outside solution (*60 cc. vol.*):

extract = 15.0
+ crys. = 2.0 } 13.0 (0.0388) γ/cc.

extract + KOH = 3.0
+ crys. = 3.0

Theoretical = 3.94 γ
Actual = 3.57 γ

b) 200 eggs (as above), ground in mortar in 60.0 cc. 0.25 N H_2SO_4; filtered; added 10 cc. 2.5 N NaAc + 1 cc. stock riboflavin (0.1 γ/cc.) + H_2O to 200 cc. total volume.

extract = 56.0
+ crys. = 9.5 } 46.5 (0.1388) γ/cc.

extract + KOH = 8.0
+ crys. = 9.5

dialyzed as in *a*

Inside of shell (*30 cc. vol.*):

extract = 31.0
+ crys. = 11.0 } 20.0 (0.0597) γ/cc.

extract + KOH = 8.0
+ crys. = 8.5

Outside solution (*60 cc. vol.*):

extract = 15.0
+ crys. = 2.0 } 13.0 (0.0388) γ/cc.

extract + KOH = 3.0
+ crys. = 3.5

Theoretical = 4.16 γ
Actual = 4.15 γ

TYPICAL DIALYSIS EXPERIMENTS

Diapause eggs (100 days old) at 25° C.: 100 eggs ground in mortar in 30 cc. 0.25 N H_2SO_4 and 4.8 cc. 2.5 N NaAc and H_2O to 100 cc.; filtered.

1. *A*. extract = 33.0 } 24.5 (0.0743) γ

B. extract + KOH = 7.0
+ crys. = 8.0

C. dialyzed *A* against 0.25 N H_2SO_4

Inside:

extract = 19.0
+ crys. = 8.0 } 11.0 (0.0334) γ/cc.

extract + KOH = 6.0
+ crys. = 6.5

Outside:

extract = 18.0
+ crys. = 2.0 } 16.0 (0.0485) γ/cc.

extract + KOH = 3.0
+ crys. = 3.5

60 days old at 25° C.: 100 eggs (as in 1)

A. extract = 26.0
+ crys. = 6.5 } 19.5 (0.0583) γ

B. extract + KOH = 6.0
+ crys. = 7.0

C. dialyzed *A* against 0.25 N H_2SO_4

Inside:		*Outside:*	
extract = 15.5 + crys. = 6.5	9.0 (0.0273) γ/cc.	extract = 14.0 + crys. = 2.0	12.0 (0.0364) γ/cc.
extract + KOH = 5.0 + crys. = 5.0		extract + KOH = 3.0 + crys. = 3.0	

Diapause eggs (90 days old) at 25° C.: 100 eggs (as in 1)

3. *A.* extract = 32.5 + crys. = 11.0	21.5 (0.0651) γ/cc.	*B.* extract + KOH = 8.5 + crys. = 9.5	

C. dialyzed *A* against 0.25 N H_2SO_4

Inside:		*Outside:*	
extract = 18.0 + crys. = 10.0	8.0 (0.0242) γ/cc.	extract = 16.0 + crys. = 3.0	13 (0.0393) γ/cc.
extract + KOH = 7.0 + crys. = 7.0		extract + KOH = 4.5 + crys. = 5.0	

43 days at 25° C.: 100 eggs (as in 1)

A. extract = 34.5 + crys. = 9.0	25.5 (0.0772) γ/cc.	*B.* extract + KOH = 7.0 + crys. = 8.0	

C. dialyzed *A* against 0.25 N H_2SO_4

Inside:		*Outside:*	
extract = 18.0 + crys. = 8.0	10 (0.0303) γ/cc.	extract = 16.0 + crys. = 2.0	14 (0.0424) γ/cc.
extract + KOH = 6.0 + crys. = 6.5		extract + KOH = 2.5 + crys. = 2.5	

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THE EFFECT OF THIOUREA ON THE OXYGEN CONSUMPTION OF FUNDULUS

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INFORMATION concerning the functions of the thyroid gland of teleosts is scanty. It is apparently concerned in the changes that occur during metamorphosis of the eel and certain flatfishes (Murr and Sklower, 1928; Von Hagen, 1936). The growth rate and body form of several species of teleosts may be materially altered by the administration of mammalian thyroid material (Harms, 1935; Lieber, 1936; Grobstein and Bellamy, 1939). Moreover, the thyroid of teleosts can apparently be activated by pituitary extracts, including those of fishes (Gorbman, 1940; Albert, 1945). But, so far, no evidence has been obtained that it plays any role in controlling general metabolic processes in these cold-blooded forms. Drexler and Von Issekutz (1935) found that neither thyroxin nor thyreotropic hormone had any effect on carbon dioxide production or oxygen consumption in guppies. Etkin, Root, and Mofshin (1940) were unable to detect any increase in oxygen consumption in goldfish that were fed thyroid tablets for as long as 9 weeks. Smith and Everett (1943) likewise failed to find any change in the oxygen consumption or growth rate of guppies fed thyroid powder. Such negative effects of thyroid administration should be supplemented by experiments which involve removal of the gland. Because of the diffuse nature of the teleost gland, complete removal is impossible. Recently, however, it has been shown that thiourea and related substances inhibit activity of the thyroid gland in amphibians, birds, and mammals. As far as fishes are concerned, Goldsmith and his co-workers (1944) have reported that, following prolonged treatment with thiourea, young hybrids of *Platypoecilus* and *Xiphophorus* showed retarded growth and failure of development of secondary sexual characters, results which they believe to be associated with impaired production of the thyroid hormone in these animals. If this is so, then the use of this drug affords an opportunity of suppressing the activity of the teleost thyroid and observing any effect that this might have on oxygen consumption. With this end in view, the following experiments were carried out.

METHODS

The experimental animals, *Fundulus heteroclitus*, were fed and maintained in the laboratory for approximately 2 weeks before using. Females ranging in weight between 6.4 and 20.2 gm. were then selected, weighed, and placed in respiration chambers of two sizes. The smaller fish were kept in bottles of 250-cc. capacity, the larger ones in 500-cc. containers. These chambers were immersed in a bath, the temperature of which was maintained between 20°.8 and 21°.4. Flow of sea water through the containers varied in the different bottles between 43 and 128 cc/minute. During the course of a day's run the flow in any one bottle varied less than 1 cc/minute. Sometimes during a 24-hour period a variation of as much as 3 cc/minute occurred, owing to

fouling of the overflow tip which regulated the flow. In these cases the tip was removed and cleaned, which re-established the former rate of flow, and the chamber was then allowed to run for an hour or more before samples were collected. Animals were kept in the chambers from 3 to 21 days, with most of the experiments running 8–10 days. During this time they received no food.

Samples of the inflow and outflow water from each chamber were taken daily, always at about the same time in the morning. The dissolved oxygen in both samples was then determined, with the Birge-Juday (1911) modification of the Winkler method. The product of the difference between these two determinations and the rate of flow gave the oxygen consumption in cubic centimeters per fish per minute. Results were expressed in these units rather than in the more general cubic centimeters per gram per minute, because considerable error in weighing an animal and its loss of weight during its stay in the chamber would render the more general units of questionable value.

The toxic dose of thiourea was determined to be about 3–4 mgm/gm. The maximum dose producing no signs of distress in practically all cases was 0.5–1.0 mgm/gm/day, and this was selected as the dosage for use in the respiratory experiments. These volumes produced no apparent injury to the swim bladder or other visceral organs. The drug was administered daily in amounts equaling 0.1–0.2 cc. and was given intra-abdominally. The injections were made after the daily water samples were drawn, so that each animal was left undisturbed for at least 24 hours before its oxygen consumption was determined. Our previous experiments on another species (*Girella nigricans*) (1942) had demonstrated that injections of sea water produce a slight rise in oxygen consumption, which lasts for only 60–90 minutes, presumably a result of handling the fishes. Since in the present experiments our samples were taken 24 hours after injection, the control animals were left untreated.

RESULTS

The oxygen consumption of *Fundulus* falls off somewhat irregularly as long as the animal is kept in the container, as illustrated in Figure 1, *A*, *B*. Even when the fish were kept in the chambers for 21 days, the oxygen consumption continued to fall fairly steadily (Fig. 1, *C*). Apparently in *Fundulus* there is no well-defined basal level; but, as long as food is withheld, the metabolism continues to decrease, as is true for a number of cold-blooded forms (Krogh, 1941, p. 7). As is shown in the figures, in most cases the determinations showed relatively high and low rates of oxygen consumption alternating on successive days. No clue to the significance of this phenomenon was obtained.

Since no basal level of metabolism could be established in these animals, thiourea injections were begun in a number of them as early as the second day in the chamber. The experiments were divided into two groups. In the first group injections were begun on the second day and continued for 5–6 days, with injections every day in some and on alternate days in others. Injections were then discontinued, and the animals were maintained in the chambers for 5 or 6 days longer. In the second group the procedure was reversed. No injections were made during the first 5 or 6 days, after which injections were made either daily or on alternate days for about 6 days. Eighteen animals in all received thiourea, and ten were left as normal controls. Four representative cases of the thiourea-treated animals are shown in Figure

2, which may be compared with the three representative cases of normal controls shown in Figure 1. Analysis of the data shows that in six animals there was a slight rise in oxygen consumption (Fig. 2, *XIIA*, *XIIIA*); in seven animals the downward slope of the curve characteristic of untreated animals was arrested while the drug was administered (Fig. 2, *XA*); and five animals showed a falling curve of oxygen consumption comparable to that of the untreated fish. Why some of the treated fishes should differ from the untreated ones in this way is not clear from these experiments; but, at any rate, it is apparent that the thiourea did not depress the oxygen consumption in these animals.

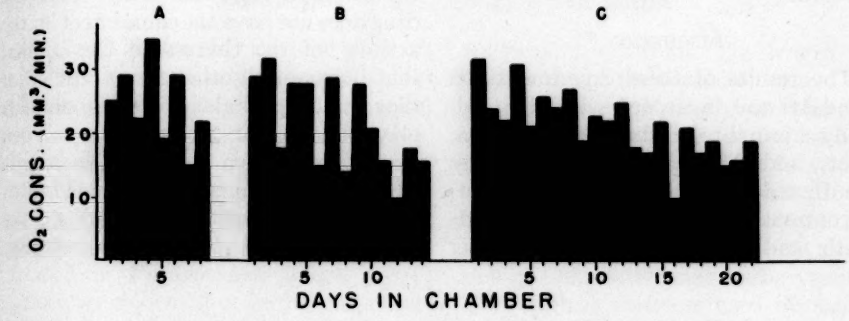

FIG. 1.—Oxygen consumption (mm.³/min.) of three untreated *Fundulus*

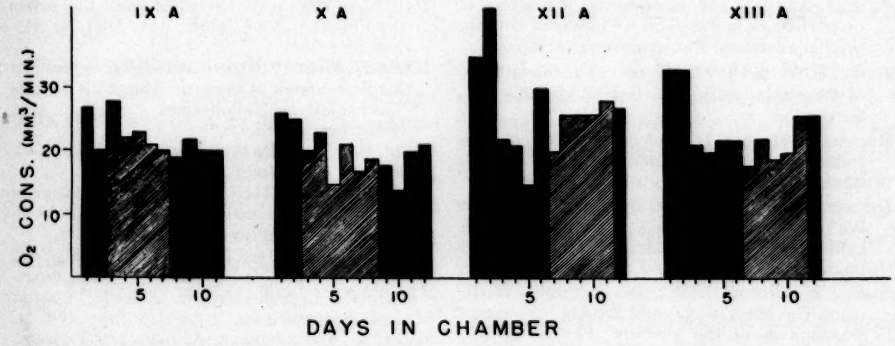

FIG. 2.—Oxygen consumption (mm.³/min.) of four representative cases of thiourea-treated *Fundulus*. Cross-hatched areas indicate time during which the animals were subjected to thiourea.

At different seasons of the year the follicles of the teleost thyroid show histological changes, which indicate considerable variation in secretory activity (Von Hagen, 1936; Hoar, 1939; Buchmann, 1940. Thus in these experiments a possible explanation for negative results is that the thyroids of the animals concerned were already in such a state of reduced activity that further reduction was impossible. If this is true, then these animals might be expected to respond to treatment with thyroid extracts. Accordingly, a series of nine animals was run for 8 days. Six individuals received injections of synthetic thyroxine (Roche-Organon, containing 10 per cent alcohol) on the second and fifth days of the run,

totaling 0.16, 0.28, and 0.5 mg. (two cases each). The other three were injected with ethyl alcohol in sea water in concentrations corresponding to those present in the three concentrations of thyroxine used. In no case was any significant change in oxygen consumption observed.

DISCUSSION

The results of these experiments on *Fundulus* are in accord with those already referred to on the goldfish (Etkin, Root, and Mofshin) and the guppy (Smith and Everett). They are, however, in contrast to those obtained by Goldsmith and his co-workers on young hybrids of a strain involving *Platypoecilus* and *Xiphophorus*. These differences may be due to differences in the species used or to age differences in the two groups, since our experiments on *Fundulus* were carried out on adult animals. The failure of thiourea to affect oxygen consumption indicates either that the drug does not have the same effect on the activity of the thyroid in this animal that it has in all other forms studied or that the thyroid gland in these animals plays no role in determining oxygen metabolism. From the negative results on oxygen consumption obtained with thyroxine by ourselves and others, the latter conclusion appears more likely.

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THE EFFECT OF SALTS AND OSMOTIC CONCENTRATION ON THE SIZE OF FOOD VACUOLES IN VORTICELLA SIMILIS

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IT IS well known that the food vacuoles formed in ciliates vary greatly in size. There is, however, little known of the factors involved in this variation. Metalnikow (1912) maintains that a rapid increase in the amount of food in suspension causes temporarily a great increase in the size of the food vacuoles formed in *Paramecium*. Frisch (1938) presents excellent, but incidental, observations on the effect of age of the culture on the size of the food vacuoles formed in *Paramecium*. Bowen (1942*a*) and Mast and Bowen (1944) show that the size of the food vacuoles of *Vorticella* is influenced by salts. This paper deals further with the effect of salts on the size of the food vacuoles in *V. similis*.

MATERIAL AND METHODS

Vorticella similis is an excellent organism in which to measure food vacuoles. The newly formed food vacuoles are spindle-shaped and are formed at the distal end of the pharynx within a cohering cluster of long fibers, which extend through the cytoplasm to the aboral end of the animal. When a vacuole forms, these fibers spread over it equidistantly from one another but at the distal end join in the cluster, thus, apparently, compressing that end to a point. When the vacuole passes into the interior, it is separated from the pharynx by a process which results in the proximal end's also being pointed. Thus, the food vacuoles are prolate spheroids. The length (the major axis) of the vacuoles, formed by a given specimen, is nearly constant, but the width (the minor axis) varies. Since the volume of spheroids is a function of the length of the axes and since, in this instance, the major axis is constant, the length of the minor axis serves as a criterion of the relative size of the vacuoles. The width is therefore used throughout this paper as a criterion of size.

The specimens of *V. similis* were obtained from laboratory cultures which consisted of two crushed hemp seeds in about 20 cc. of boiled tap water in a Petri dish. A few short hairs were added to each culture to serve as a place of attachment for the vorticellae in the culture and while being studied on the slide.

The observations were made as follows: A hair with vorticellae attached was transferred to a drop of culture fluid on a slide between two parallel ridges of vaseline. The preparation was covered with a cover glass, and a specimen favorable for observation was selected. The width of several consecutive food vacuoles was then measured at the time they left the pharynx. The measurements were made with a 10× micrometer ocular and a 1.6-mm. oïl-immersion objective. If vacuoles of fairly constant size were being formed in regular succession, the culture fluid was replaced with water redistilled in Pyrex glass, and a number of consecutive vacuoles were measured. Then the distilled water was replaced with a weak solution of salt, and again a

[1] Now at the National Institute of Health, Bethesda 14, Maryland.

number of consecutive vacuoles were measured. This was repeated with stronger solutions with the same specimen. A number of specimens were thus studied in each of a series of concentrations of NaCl, KCl, and $CaCl_2$.

The solutions under the cover glass were changed by putting several drops of solution at one end of the cover glass and a long strip of filter paper at the other end. The latter pulled the solution under the cover glass and carried it over the edge of the microscope stage. More fluid was added as needed. The current of fluid extended the specimens and kept them continuously in the same position except, occasionally, when they contracted. At those times they usually extended immediately and were pulled back to the same position by the current. For this reason and others, such as maintaining adequate oxygen, all measurements were made with the fluid flowing past the specimens. In this manner measurements of food vacuoles formed by a single specimen were made during several hours in several consecutively applied concentrations of a salt. The number of such concentrations in which measurements were made varied because of detachment or onset of fasting of the specimen. Each specimen used in making measurements in distilled water was used again in the respective salt solutions. The results obtained in the studies of NaCl, KCl, and $CaCl_2$ are presented in Table 1.

From 4 to 12 food vacuoles were measured in each specimen in the study of the effects of NaCl, KCl, and $CaCl_2$. The number of measurements in each concentration, therefore, equals four to twelve times the number of specimens subjected to the given concentration. The mean width of the food vacuoles formed by each of several specimens in a given solution was calculated. Then the mean of the means in the several specimens was calculated and is presented in Table 1 as the mean width. The mean width, in other words, is the average of all the vacuoles measured under each condition in a series of specimens.

The probabilities that the differences between the means occurred by chance were obtained by use of the *t*-test of "Student" and the probability table of Fisher and Yates. In the tables, *D* is the difference between the mean widths, and *P* is the probability that the differences occurred by chance.

THE EFFECTS OF SODIUM, POTASSIUM, AND CALCIUM

The food vacuoles formed in all the concentrations tested of NaCl, KCl, and $CaCl_2$ were smaller than those formed in distilled water. As the concentration of NaCl and KCl was increased, the size of the food vacuoles decreased throughout the range which could be tested. As the concentration of $CaCl_2$ was increased, the vacuoles first decreased in size and then increased (see Table 1). The decreases in vacuolar size in the solutions of NaCl and $CaCl_2$ were of about the same magnitude, but the decreases in KCl were considerably greater than in either of the others. The facts that, as the concentration increased, the vacuoles formed in $CaCl_2$ decreased to a minimum and then increased and that at the same concentration the effect of KCl was much greater than that of NaCl indicate that the observed action of the salts was correlated with their cations. The fact that the effect of the three chlorides differed strengthens this conclusion.

The increase in size of the vacuoles in the higher concentrations of $CaCl_2$ is, according to Table 1, correlated with the increase in concentration; but the following results indicate otherwise: In the experiments of Table 1 the specimens were studied about 30 minutes in each con-

centration, so that, by the time they were put into 10^{-3} M and 10^{-2} M $CaCl_2$, they had been subjected to $CaCl_2$ anywhere from 30 to 90 minutes, depending upon whether they were put in 10^{-5} M (only four specimens were studied in 10^{-5} M). In other experiments specimens were subjected to 10^{-2} M $CaCl_2$ directly after having been studied in distilled water. In the latter, 10 food vacuoles averaged 8.1 μ in width in one specimen and 9.2 μ in a second. In 10^{-2} M $CaCl_2$ the vacuoles averaged 6.8 μ in the first specimen and 6.6 μ in the second. These results show that, when not preceded by prolonged exposure to lower concentrations, higher concentrations of $CaCl_2$ cause a decrease in size of food vacuoles, as do lower concentrations, and they make it appear likely that the increase in size shown in Table 1 is attributable to the adjustment of *Vorticella* to calcium.

The concentration of salt in which *Vorticella* stops formation of food vacuoles was not carefully ascertained; however, three specimens were subjected to

TABLE 1

EFFECTS OF SALTS IN DIFFERENT CONCENTRATIONS ON SIZE* OF THE FOOD VACUOLES FORMED BY *Vorticella similis*

No. of Vorticellae Subjected to Both Conditions†	Molar Concentration in Distilled Water				*D*	*P*
	0	1.4×10^{-4}	1.4×10^{-3}	1.4×10^{-2}		
	NaCl					
14	10.9	9.8	.....	.....	1.1	<.02
15	11.1	.....	8.8	.....	2.3	<.001
15	11.1	.....	.....	8.4	2.7	<.001
	KCl					
5	10.4	8.2	.....	.....	2.2	<.05
4	10.4	.....	5.5	.....	4.9	<.001

No. of Vorticellae Subjected to Both Conditions†	Molar Concentration in Distilled Water					*D*	*P*
	0	10^{-5}	10^{-4}	10^{-3}	10^{-2}		
	$CaCl_2$						
4	10.4	9.2	.....	.....	.....	1.2	>.05
9	10.6	.....	7.3	.....	.....	3.3	<.001
13	10.1	.....	.....	7.9	.....	2.2	<.001
12	9.8	.....	.....	.....	8.0	1.8	<.001
9	.....	.....	7.4	8.5	.....	1.1	>.05
7	.....	.....	7.0	.....	8.4	1.4	<.01

* Mean width in micra.

† The numbers shown in this column represent the number of specimens of the total used which were subjected to the two conditions being compared. The total for NaCl is 15, for KCl 5, and for $CaCl_2$ 16.

2.8×10^{-2} M NaCl, and two formed food vacuoles normally. The third stopped feeding. Two specimens were subjected to 7×10^{-2} M, and both stopped feeding. Three specimens were subjected to either 7×10^{-3} or 1.4×10^{-2} M KCl. All stopped feeding. Seven specimens were subjected to 2×10^{-2} M $CaCl_2$ after exposure of about 2 hours to lower concentrations. Of these, four formed food vacuoles normally, and three stopped feeding. The mean width of the food vacuoles of the four specimens was 8.0 μ.

THE EFFECT OF INCREASED OSMOTIC PRESSURE

The decrease in the size of the food vacuoles formed in solutions of NaCl, KCl, and $CaCl_2$ appears to be caused by the cations involved and not by the increases of osmotic pressure. More direct evidence, however, on the effect of increased osmotic pressure was obtained by subjecting *Vorticella* to lactose in distilled water and in boiled tap water. The experiments were done and the measurements treated by the same methods as were those with the salts. From 10 to 20 food vacuoles were measured in each specimen. The number of measurements in each concentration, therefore, equals ten to twenty times the number of specimens subjected to the given concentration. The results obtained are presented in Table 2.

The food vacuoles formed in the three concentrations of lactose in distilled water and in 6.25×10^{-3} M lactose in tap water were neither reliably smaller nor reliably larger than those formed in distilled water and tap water, respectively. Those formed in 1.25 and 2.5×10^{-2} M lactose in tap water, however, were reliably smaller than those formed in tap water (Table 2).

The results obtained from the specimens in solutions of lactose in distilled water strongly support the contention that the increases of the osmotic pressure accompanying the additions of salt to distilled water were not involved in the effect of those solutions on the size of the food vacuoles. The results obtained in the solutions of lactose in tap water complicate this picture by making it appear that the salts of tap water modify the noneffectiveness of uncomplicated increases of osmotic concentration. The results of the following section have some bearing on this possibility.

THE EFFECT OF $CaCl_2$ IN ISOSMOTIC SOLUTION

In the experiments described above, in which lactose was added to tap water, the osmotic concentration was varied, but the molecular concentration of the natural and other salts remained fairly constant. In the next series of experiments the concentration of salt was varied, and the osmotic concentration was maintained constant by adding lactose to each solution of salt sufficient to make the osmotic concentration equal to that of the highest concentration of salt tested. Only experiments with $CaCl_2$ were completed. The osmotic concentration of each solution was 0.68 atm.

The experiments were done, and the measurements were treated by the same methods as those with the salts. From 5 to 11 food vacuoles were measured in each specimen. The number of measurements in each condition, therefore, equals five to eleven times the number of specimens subjected to the given condition. The molecular concentrations of lactose and $CaCl_2$ in each solution and the results of the experiments are given in Table 3.

Table 3 shows that the relation between the increases in concentration of $CaCl_2$ and the size of the vacuoles was

similar to, but not so great as, that in the solutions of $CaCl_2$ of varying osmotic concentration. These results support the above contention that the action of NaCl, KCl, and $CaCl_2$ was not caused by osmosis but by the cations.

The conflicting results given in Table 2 can now be considered in the light shed by those given in Table 3. The fact that the greatest difference in size shown in Table 3 occurred in 10^{-3} M $CaCl_2$ possibly lends credence to the contention in the preceding section that the salts of tap water modified the noneffectiveness of simple increases of osmotic concentration. It is known that calcium, as hy-

TABLE 2

RELATION BETWEEN OSMOTIC CONCENTRATION AND SIZE* OF FOOD VACUOLES FORMED BY *Vorticella similis*

No. of Vorticellae Subjected to Both Conditions†	Molar Concentration of Lactose in Distilled Water				*D*	*P*
	0	6.25×10^{-3}	1.25×10^{-2}	2.5×10^{-2}		
6	11.0	12.0	.....	.....	0.1	>.05
8	11.7	.....	11.2	.....	0.5	>.05
5	10.8	.....	.....	10.8	0.0	.....

No. of Vorticellae Subjected to Both Conditions†	Molar Concentration of Lactose in Tap Water				*D*	*P*
	0	6.25×10^{-3}	1.25×10^{-2}	2.5×10^{-2}		
7	9.7	9.4	.....	.....	0.3	>.05
8	9.4	.....	8.5	.....	0.9	<.05
7	9.7	.....	.....	8.2	1.5	=.01

* Mean width in micra.

† The numbers shown in this column represent the number of specimens of the total used which were subjected to the two conditions being compared. The total for each of the distilled water and the tap-water series is 8.

TABLE 3

RELATION BETWEEN ISOSMOTIC CONCENTRATIONS OF $CaCl_2$ AND SIZE* OF FOOD VACUOLES FORMED BY *Vorticella similis*

No. of Vorticellae Subjected to Both Conditions†	Molar Concentration of $CaCl_2$ in Lactose Solution‡						*D*	*P*
	Dist. H_2O	0	10^{-4}	10^{-3}	5×10^{-3}	10^{-2}		
6	9.8	9.7	.....	.....	.....	.....	0.1	>.05
4	9.4	.....	8.6	.....	.....	.....	0.8	>.05
4	9.6	.....	.....	8.5	.....	.....	1.1	=.01
4	9.6	.....	.....	.....	8.0	.....	1.6	>.05
4	9.6	.....	.....	.....	.....	9.1	0.5	>.05

* Mean width in micra.

† The numbers shown in this column represent the number of specimens of the total used which were subjected to the two conditions being compared. The total is 6.

‡ The molarity of lactose necessary to make the osmotic concentration of each concentration of $CaCl_2$ equal to that of 10^{-2} M $CaCl_2$ is as follows: 0 M, 2.6×10^{-2} M; 10^{-4} M, 2.53×10^{-2} M; 10^{-3} M, 2.33×10^{-2} M; 5×10^{-3} M, 2.06×10^{-2} M; 10^{-2} M, none.

drated lime, was present in the tap water used in the experiments of the preceding section in amounts sufficient to yield concentrations as high as 3×10^{-4} M. The results given in Table 1 indicate that this concentration of calcium, at least as $CaCl_2$, would cause a decrease in size of the food vacuoles; they also indicate that vorticellae exposed to a certain concentration for a long period become less sensitive to calcium. These organisms were reared in tap water. The concentration of calcium in the solutions of lactose in tap water was constant. These considerations call attention to the unexplored possibility that increases of osmotic concentration do affect the size of the food vacuoles under certain chemical or physical conditions.

Other evidence, however, indicates that simple osmotic concentration does not influence the size of the food vacuoles of *Vorticella*. This evidence is: (1) that, as the duration of exposure increased, $CaCl_2$ effected a smaller decrease in size of the vacuoles, in spite of increases of both the molecular and the osmotic concentration; (2) that at the same concentration the effect of KCl was much greater than that of NaCl; (3) that increases of osmotic concentration produced by adding lactose to distilled water did not affect the size of the food vacuoles.

DISCUSSION

Such studies as these may be of value in ascertaining the nature of intracellular membranes such as occur in the Protozoa. Since such membranes are inaccessible to direct study, indirect evidence like the foregoing must be gathered and analyzed to understand their nature. Other work interrupted this series of experiments, so the picture regarding the food-vacuolar membranes of *Vorticella* is far from complete; however, some analysis can be made.

The width and consequently the size of the food vacuoles in *Vorticella* are affected by the concentration of the cations present. Is this effect because of changes in the elasticity and rigidity of the vacuolar membrane, changes in the viscosity of the cytoplasm, or a decrease in the cohesiveness of the esophageal fibers? The last alternative is highly doubtful because such a decrease would be manifested by a lengthening of the food vacuoles as well as by a widening. Increased length of the vacuoles was not observed to any extent. Consequently, the cations must exert their effect by one of the first two alternatives or by both.

Bowen (1942*b*) presents data which indicate that the decrease in the size of the vacuoles is because of a change in the physical properties of the vacuolar membrane and not of the cytoplasm. He found that NaCl or $CaCl_2$ sometimes affects the width of the food vacuoles of *Vorticella* within 30 seconds after the salt is applied. He believes 30 seconds to be too little time for dispersal of the salt through all the cytoplasm of this organism. This is undoubtedly true, but it is enough time for the cations to pass into the cytoplasm adjacent to the forming vacuole. Alteration of the cytoplasm of that region might be sufficient to change the properties of the vacuolar membrane or to compress the vacuole. The data in Table 1, however, do not support this hypothesis.

Table 1 shows that, when time is allowed for the salts to disperse through all the cytoplasm of the vorticellae, specimens in calcium solutions form larger vacuoles than when first exposed, and those in sodium and potassium continue to form small vacuoles. These specific actions of the cations are informatory. Both species of cations have the same

qualitative action on the food-vacuolar membrane, but opposite actions upon any likely cytoplasmic influence in vacuolar formation. There is no obvious reason why the salts should affect the cytoplasm throughout the cell differently from that adjacent to the forming food vacuole. These consequences indicate strongly that the effect of electrolytes upon the size of the food vacuoles in *Vorticella* is not attributable to indirect action through the cytoplasm but to direct action upon the vacuolar membrane. Further studies of this problem are desirable and may contribute some evidence necessary for determining whether vacuolar membranes are akin to morphological structures or are simply interfaces between two kinds of matter.

SUMMARY

1. *Vorticella similis* forms spindle-shaped food vacuoles, which, in a single specimen, vary in width but not appreciably in length. The width can therefore be used as a criterion of the variation in size.

2. NaCl, KCl, and $CaCl_2$ cause a decrease in the size of the food vacuoles.

3. The size of the food vacuoles formed in solutions of NaCl and KCl varies inversely with concentration.

4. The size of the food vacuoles formed in solutions of $CaCl_2$ decreases to a minimum and then increases as the concentration increases.

5. In solutions of $CaCl_2$ made isosmotic with lactose, the size of the food vacuoles decreases to a minimum and then increases as the concentration of $CaCl_2$ increases, as it does in pure solutions of $CaCl_2$ but not to the same extent.

6. The size of the food vacuoles formed in solutions of lactose in distilled water is not correlated with osmotic concentration.

7. The action of these salts on the size of food vacuoles is attributable to their cations, probably acting directly on the vacuolar membrane.

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AN ANALYSIS OF PHOTOELECTRIC INSTRUMENTS FOR MEASUREMENT OF TURBIDITY WITH REFERENCE TO SEROLOGY[1]

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THE recent application of photoelectric methods (Boyden, 1942; DeFalco, 1941, 1942; Baier, 1943) to studies in serology for the determination of the degree of turbidity in the reaction mixture would indicate the desirability of a review of the theoretical problems involved and a study of basic instrument performance.

Early studies to determine the strength of precipitin reactions employed the flocculation test. The ring test soon replaced the flocculation test as a technique when it was found that the "titer," or end-point of the latter, was difficult to determine. Likewise, the ring test is being augmented by methods which indicate the nature of the reaction throughout the antigen dilution series, instead of indicating only that highest dilution of the antigen in which a definite ring of precipitate is still visible. These methods of total precipitate measurement are varied, including the determination of (1) the dry weight, (2) the nitrogen content of the washed precipitates (Wu, Cheng, and Li, 1928; Heidelberger *et al.*, 1929–43), and (3) the volume of the precipitate (Boyden and Baier, 1929; Baier, 1933).

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Studies of the dry weight and the nitrogen content of serological precipitate have not been used to any great degree in the study of animal relationships. They have been applied more to a study of the theoretical aspects of precipitate formation in the precipitin reaction itself (see Heidelberger *et al.*).

One technique for precipitate volume measurement employs the centrifuge (Boyden and Baier, 1929) to pack the precipitate in calibrated capillary tubes. While some studies (Boyden, 1934; Baier and Wolfe, 1942) on animal relationships have been made with this technique and its statistical reliability has been established, the use of this procedure is time-consuming and will probably be abandoned for that reason alone. The techniques which will replace it are ones to determine the degree of the reaction by measuring the total turbidity by means of photometric principles (Libby, 1938; Baier, 1943).

For that reason it seems necessary that a clear understanding of the nature of the turbidimetric problems be reviewed (see Yoe, 1928; Müller, 1939; Gibb, 1942), so that proper comparisons of the instrumental data can be made in the study of animal relationships.

THEORETICAL CONSIDERATIONS

When a turbid system is illuminated by a beam of light, (1) a portion of the incident light is reflected by the particles, (2) a portion is absorbed by the suspended particles and by the solution, and (3)

a portion of the incident light is transmitted through the solution.

Measurements of turbidity can be carried out in any of four ways: (1) by measuring the transmitted light; (2) by measuring the scattered light; (3) by expressing turbidity in terms of the ratio of scattered light to the transmitted light; and (4) by methods of determining complete extinction of the turbid mixture. The first two methods are being used in serological studies.

A. TRANSMITTED LIGHT MEASUREMENTS

Essentially, the theoretical aspects of the measurement of turbidity by this method find an explanation in the same laws which govern the absorption of light by colored solutions, provided that the particles causing the turbidity are of uniform size and are evenly dispersed throughout the medium. The measurement of turbidity by this means is on the basis of the Bouger-Beer law applied to turbid systems, where (Fig. 1, *A*)

$$I = I_0 e^{-kct}$$

when using monochromatic light. In the formula, I_0 is the incident light, and I is the emergent light of the cell, containing a solution of thickness t and of concentration c, and k is a constant for the physical setup of the apparatus.

If c is expressed in moles per liter and t is in centimeters, then k is the molecular extinction coefficient; I/I_0 is the transmission, T; and $\log_{10} I_0/I = E$, the extinction. It follows that $E = kct$ and $-\log T = E$. If the usual visual photometer of the Duboscq type is used, the depths, t, of the two solutions to be compared (Fig. 1, *B*) are varied until a density match is obtained, when

$$E_1 = E_2 \quad \text{or} \quad I_1 = I_2$$

and

I_0 = Constant illumination;

or

$$k c_1 t_1 = k c_2 t_2 ,$$

and

$$\frac{C_1}{C_2} = \frac{t_2}{t_1}.$$

In photoelectric densitometers the procedure is altered in that the thickness, t, of the solutions to be compared is kept constant and I_1 and I_2 are measured instead, where

$$I_1 = I_0 e^{-kc_1}$$

and

$$I_2 = I_0 e^{-kc_2} ;$$

therefore,

$$\log \frac{I_1}{I_2} = k\,(c_2 - c_1) .$$

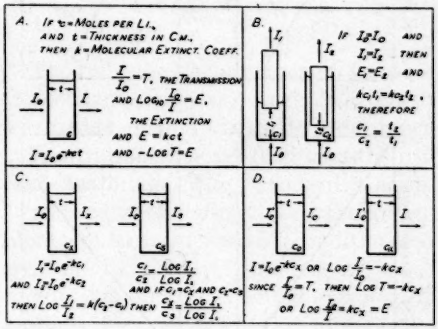
A.
IF c = MOLES PER LIT.
AND t = THICKNESS IN CM.
THEN k = MOLECULAR EXTINCT. COEFF.
$\frac{I}{I_0} = T$, THE TRANSMISSION
AND $\text{LOG}_{10}\frac{I_0}{I} = E$,
THE EXTINCTION
AND $E = kct$
AND $-\text{LOG}\,T = E$
$I = I_0 e^{-kct}$
B.
IF $I_0 = I_0$ AND
$I_1 = I_2$ THEN
$E_1 = E_2$ AND
$kc_1 l_1 = kc_2 l_2$.
THEREFORE
$\frac{c_1}{c_2} = \frac{t_2}{t_1}$
C.
$I_1 = I_0 e^{-kc_1}$
AND $I_2 = I_0 e^{-kc_2}$
THEN $\text{LOG}\frac{I_1}{I_2} = k(c_2 - c_1)$
THEN $\frac{c_1}{c_2} = \frac{\text{LOG}\,I_1}{\text{LOG}\,I_2}$
AND IF $c_1 = c_x$ AND $c_2 = c_s$
$\frac{c_x}{c_s} = \frac{\text{LOG}\,I_x}{\text{LOG}\,I_s}$
D.
$I = I_0 e^{-kc_x}$ OR $\text{LOG}\frac{I_x}{I_0} = -kc_x$
SINCE $\frac{I_x}{I_0} = T$, THEN $\text{LOG}\,T = -kc_x$
OR $\text{LOG}\frac{I_0}{I_x} = kc_x = E$

FIG. 1.—Theoretical aspects of photometry

This can be done by either of two methods.

1. Comparison with a standard solution (Fig. 1, *C*), where

$$\frac{c_1}{c_2} = \frac{\log I_1}{\log I_2};$$

and if

$$c_1 = c_x \text{ (unknown solution)}$$

and

$$c_2 = c_s \text{ (standard solution)} ,$$

then

$$\frac{c_x}{c_s} = \frac{\log I_x}{\log I_s}.$$

2. Comparison with a control (pure solvent, etc.) in one of the cells, which simplifies the procedure as (Fig. 1, *D*) the

intensity, I, which emerges from the cell of concentration, c_x, will be given as

$$I = I_0 e^{-kc_x}$$

or

$$\log \frac{I}{I_0} = -kc_x .$$

Since I/I_0 = Transmittancy, T, then $\log T = -kc_x$ or

$$\log \frac{I_0}{I} = kc_x = E .$$

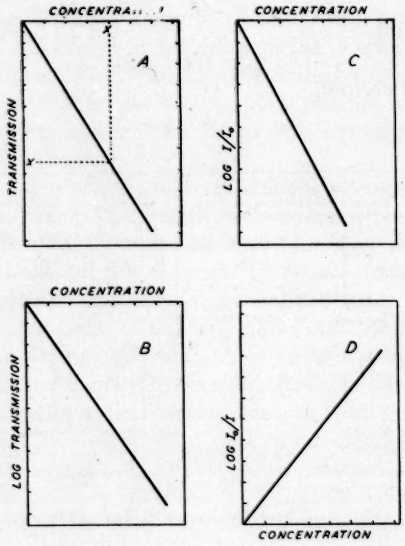

FIG. 2.—Methods of plotting data

Figure 2 illustrates several methods of graphically plotting the data. Where the instrument is designed to read light transmission in percentages from 100 to 0 and is adjusted to 100 for the transmittancy of the control cell, then, if the unknown solution, c_x, is placed in the path of light, the transmission will decrease to some value T_x. This is the transmittancy for that particular concentration and gives the ordinate value for graphical representation. All other values of T will be a linear function of c on this semilogarithmic scale if the system obeys the Bouguer-Beer law.

The microdensitometer,[2] described by Baier (1943), operates on the principles just described. The scale is calibrated in light transmission from 100 to 0, and the readings so obtained are plotted on semilogarithmic paper as indicated in Figure 2, *A*. The data could just as well be plotted as illustrated in Figure 2, *B*, *C*, and *D*. Data presented in the paper describing the instrument show that the instrument is basically sound in principle and operation. A calibration curve presented for copper sulphate, a colored solution, shows a linear relationship between concentration and light transmission (monochromatic light) when the data are plotted on semilogarithmic paper. Evidence also is presented to illustrate that the Bouguer-Beer law can be applied to turbid systems, since the same inverse logarithmic relationship of light transmission to concentration was shown for a series of dilute India ink solutions.

Since turbidity bears a direct relationship to concentration, a comparison of turbidities must be made on the basis of a comparison of the inverse logarithms of the light transmission, *not* on the basis of a comparison of light-transmission values. In other words, turbidity comparisons from the graphical data must be made on the basis of the ordinates of the readings, using a linear scale, *not* the logarithmic scale. A linear scale is included to the right of Figure 2, *A*, for this purpose. This will be illustrated in the experimental data (see Fig. 14) for an application of this instrument to studies in serology.

B. SCATTERED LIGHT MEASUREMENT

These instruments measure the light scattered by the finely divided particles

[2] This instrument has been assigned to the Wisconsin Alumni Research Foundation, who, in turn, have assigned manufacturing, use, and selling rights to the Precision Scientific Company of Chicago.

in the turbid system, with mechanisms to prevent excitation of the instrument by the transmitted light. They are often called "Tyndallometers," since they measure only the scattered light of the so-called Tyndall beam. If the scattered light is compared with a suitable reference standard, the procedure is called "nephelometry," and the instruments, "nephelometers."

Instruments which measure scattered light are admittedly very sensitive for small amounts of turbidity. But measurements of scattered light from the Tyndall beam are most critical. The measurement of turbidity by this method is on the basis that, theoretically, there is a linear relationship between the amount of suspended matter and the intensity of the Tyndall beam. As given by Rayleigh (from Müller, 1941), the intensity of the scattered light is

$$\frac{I}{I_0} = K \frac{N v^2}{\lambda^4} \sin^2 \alpha ,$$

where I is the intensity of the scattered light measured at an angle, α, to the incident beam, I_0; N is the number of particles of a volume, v; and λ is the wave length of the light. If colored dispersions are involved, the problem of selective light absorption further complicates the procedure. Actually, the linearity between the intensity of light from the Tyndall beam and the amount of suspended matter holds moderately well within narrow limits when the sample is colorless and faintly turbid. The relationship of intensity to concentration is much more complex at higher concentrations of suspended matter, as well as when the solution is colored. One of the most disturbing factors is this concomitant reduction in the intensity of light from the Tyndall beam by secondary absorption. This secondary absorption effect may be so great that increasing turbidity may actually give a lower intensity to the emergent Tyndall beam (Yoe, 1928).

Libby (1938) constructed the photronreflectometer, an instrument operating on the principle (Freygang, 1931) of determining turbidity by measuring the light scattered by the Tyndall beam referred to a standard of illumination. In the article reporting its construction, data for a standard barium sulphate suspension were presented, which, in the main, indicate a linearity between turbidity and light-scattering measurements over the range of turbidity reported.

EXPERIMENTAL MATERIALS, PROCEDURE, AND DATA

To study the performance of the photoelectric instruments, each instrument was tested against suspensions of known concentration of several differently colored materials. The standard suspensions used were chosen to test the instruments under most variable conditions, as well as to simulate conditions which might be met in actual serological studies.

The suspensions used were fine aqueous suspensions of (1) lead chromate (a yellow precipitate), (2) a red suspension of non-waterproof ink, (3) India ink (black), and (4) the white precipitates of aluminum hydroxide and barium sulphate. To illustrate the use of the instruments in serological studies, data were obtained by reacting an antiserum against its homologous antigen. The results obtained are illustrated in Figures 3–14, which follow.

The photoelectric instruments used for measurement of turbidity were (1) the Coleman spectrophotometer, Model 11; (2) the Libby photronreflectometer; (3) the Baier microdensitometer; and these instruments were compared with a technique for direct volume measurement

using (4) the Van Allen thrombocytocrits. The sample cells supplied with the photronreflectometer were replaced with ones of similar dimensions but of precision manufacture (Klett Manufacturing Co., Cell No. 1002-U, size 30 × 20 × 5 mm. I.D.). A slight modification in the instrument was necessary to accommodate the cells by simply inserting an adapter to reduce the depth of the cell-holder.

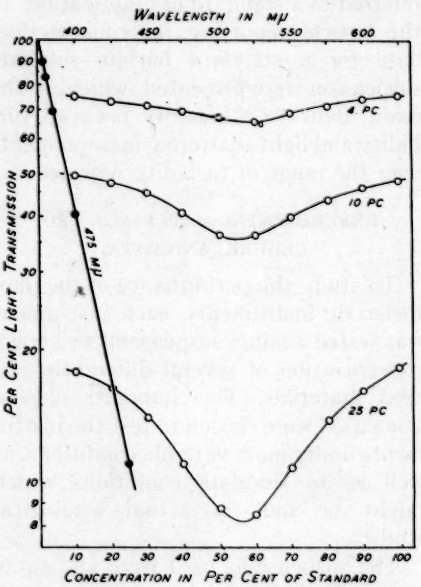

FIG. 3.—Data obtained with the Coleman spectrophotometer, using a red suspension. The solid-circle graph illustrates the relationship between transmission and concentration. The open-circle graphs show the relationship between transmission and wave length of incident light for three different concentrations.

The data illustrated in Figures 3, 4, and 5 were obtained from dilutions of the red suspension of non-waterproof ink. This standard suspension, referred to as a concentration of 100 per cent, was determined by trial so as to cover the usable instrument range for the least sensitive instrument being studied. For all transmittancy studies in the Coleman spectrophotometer and the microdensitometer, suspensions of concentrations beyond those indicated in the graphs were read in the instruments. However, the readings when plotted would not alter the linear nature of the graphs and were omitted only because, in practice, those concentrations giving a transmittancy of less than 10 per cent are ordinarily not reported for instruments of this type. Multiple-cycle semilogarithmic paper could be used if it is desired to record these data.

In Figure 3, data are plotted as obtained in the Coleman instrument, which measures the amount of light transmitted by the suspension at any selected wave length. The solid-circle graph illustrates that there is a direct relationship between concentration of suspended particles and light absorption when the data are plotted on semilogarithmic paper. While only the 475-mμ absorption curve is shown, the results at other wave lengths were also linear but of different slopes, indicating varying sensitivities at different wave lengths of incident light. The open-circle graphs are data of three different suspension concentrations plotted against variations in the wave length of the incident light. It can be seen from these graphs that the greatest absorption of light occurs at a wave length of approximately 515 mμ, and for greatest sensitivity this wave length should be chosen for studies on this particular red suspension. Figure 4 illustrates the results obtained in the same fashion with the microdensitometer. It essentially parallels the studies with the Coleman spectrophotometer. One difference is to be noted: It is less sensitive than the Coleman instrument, owing to the use in this particular series of studies of the smaller 75 × 10-mm. round absorption test tubes instead of the larger rectangu-

lar absorption cells, which, however, are available for use in the microdensitometer. These small tubes were used because they are the type usually employed in serological work where conservation of serological reactants is an important consideration.

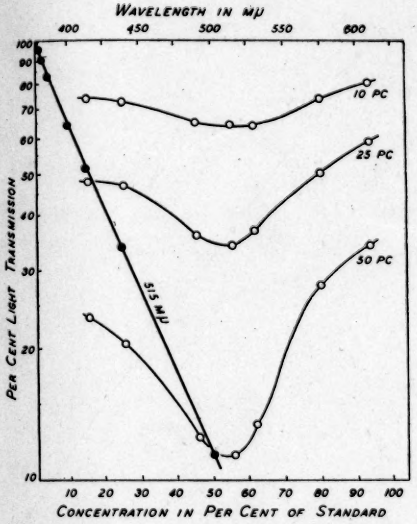

FIG. 4.—Data obtained with the microdensitometer, using a red suspension. The solid-circle graph illustrates the relationship between transmission and concentration. The open-circle graphs show the relationship between transmission and wave length of incident light for three different concentrations.

In Figure 5 are shown the data using the photronreflectometer as a means of measuring the light scattered by the suspended particles in various concentrations of the red suspension. In obtaining this series of graphs, the same turbidity samples were measured with the instrument adjusted for several sensitivities by having the instrument reference light beam preset for a maximum galvanometer reading of 50, 25, and 10. The greatest galvanometer deflection per concentration and therefore the greatest sensitivity were obtained with the reference light beam adjusted for a galvanometer deflection of 50. Since full scale on the galvanometer was 60, the instrument could have been adjusted initially at this reading for still greater sensitivity. It will be noted from the graphs that the data are essentially linear for each sensitivity setting, up to a galvanometer reading slightly in excess of the sensitivity setting. Beyond this region there is a flattening-out of the curve and eventually

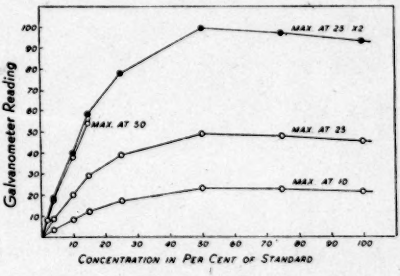

FIG. 5.—Data obtained with the photronreflectometer, using a red suspension. The open-circle graphs illustrate the data obtained with the instrument galvanometer preset for maximum deflections of 10, 25, and 50. The solid-circle graph presents the data of the curve obtained with the galvanometer preset at a sensitivity of 25, with all values multiplied by a factor of 2.

a decrease in galvanometer reading for increase in suspension concentration. This clearly indicates that the instrument cannot be used to measure the turbidity of this suspension beyond a certain maximum concentration. This nonlinearity beyond a certain concentration may result from secondary scattering of light by the too adjacent particles in the suspension.

The solid-circle graph of Figure 5 is included to illustrate an attempt at converting the readings obtained at a sensitivity of 25 to those obtained at a sensitivity of 50 by multiplying all readings of the lower sensitivity setting by a factor of 2. It will be observed that there is a slight divergence of the graphs for those

lower concentrations where comparison is possible. It will also be observed that only for low concentrations will the galvanometer readings, for the graph obtained with the instrument preset at a sensitivity of 25, be approximately two and a half times the respective readings obtained with the instrument preset for a sensitivity of 10. This procedure was studied because it is the practice, occasionally, to lower the sensitivity of the instrument so that suspensions of higher turbidity will give a reading within the range of the galvanometer scale, even though these readings are higher than the sensitivity reference maximum. These studies indicate that this practice should be followed with caution. At least, with this type of suspension and for the particular photronreflectometer used in this study, it is obvious that there is a direct linear relationship between instrument reading and turbidity only up to a certain maximum concentration of the suspension. The procedure of adjusting the instrument sensitivity by varying the intensity of light with the resultant change in spectral emission, as well as the nonuse of light filters, may be involved in this nonlinearity.

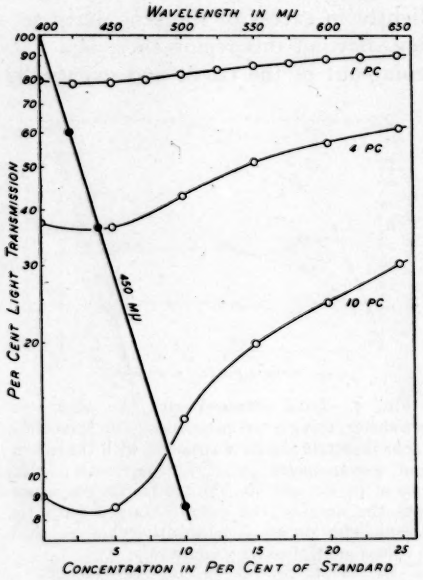

FIG. 6.—Data obtained with the Coleman spectrophotometer, using a suspension of precipitated lead chromate. The solid-circle graph illustrates the relationship between transmission and concentration. The open-circle graphs show the relationship between transmission and wave length of incident light for three different concentrations.

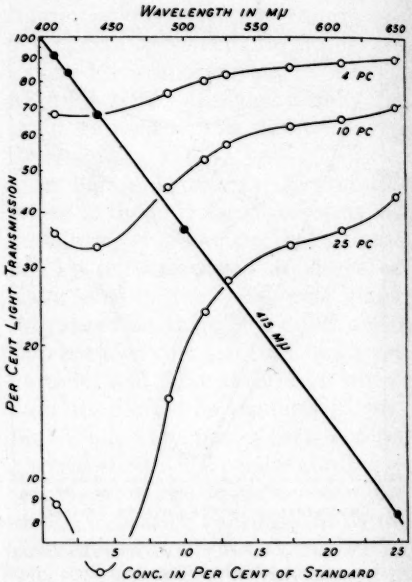

FIG. 7.—Data obtained with the microdensitometer, using a suspension of precipitated lead chromate. The solid-circle graph illustrates the relationship between transmission and concentration. The open-circle graphs show the relationship between transmission and wave length of incident light for three different concentrations.

Figures 6, 7, and 8 express data obtained by using the yellow suspension of lead chromate. Again the linearity between concentration and light absorption is seen in Figures 6 and 7 with the data for the Coleman spectrophotometer and the microdensitometer plotted on semilogarithmic paper, while Figure 8 illus-

trates the same conditions described previously for Figure 5 with the photronreflectometer.

In Figure 9 are presented data on the use of a suspension of India ink (black). Both the Coleman instrument (solid and open circles) and the microdensitometer (half-solid circles) show the same relationship between concentration and percentage light transmission as that discussed previously for the other suspensions and reported by Baier (1943) for India ink.

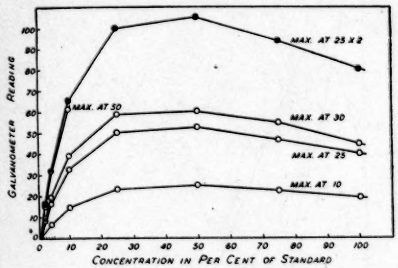

FIG. 8.—Data obtained with the photronreflectometer, using a suspension of precipitated lead chromate. The open-circle graphs illustrate the data obtained with the instrument galvanometer preset for maximum deflections of 10, 25, 30, and 50. The solid-circle graph presents the data of the curve obtained with the galvanometer preset at a sensitivity of 25, with all values multiplied by a factor of 2.

When the India ink was placed in the sample cells of the photronreflectometer, there was no observable reading on the galvanometer for the lower concentrations, but a slightly negative reading for higher concentrations. This indicates that the black-ink particles absorbed almost all the light incident upon them. What little scattering did occur was further absorbed by other ink particles in the suspension.

Suspensions of all three precipitates were also placed in the Van Allen thrombocytocrits and centrifuged at 2,400 R.P.M. for 30 minutes according to the procedure recommended by Boyden and Baier (1929). In each instance the volume of suspended matter in 2 ml. of the standard was not measurable, being less than 0.0002 ml., the smallest measurable quantity for these tubes. For this technique greater suspension concentrations would have to be employed.

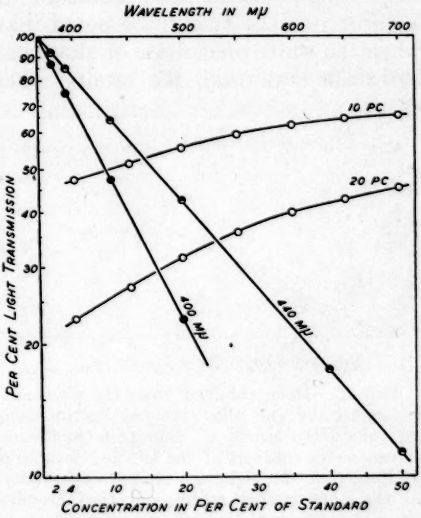

FIG. 9.—Data obtained using a suspension of India ink. The solid-circle (data of Coleman instrument) and half-solid-circle (data of microdensitometer) graphs illustrate the relationship between concentration and transmission for two different wave lengths of incident light. The open-circle graphs show the data of variations in light transmission and wave length of incident light for two different concentrations as obtained with the Coleman spectrophotometer.

Standard suspensions of barium sulphate and aluminum hydroxide were studied in the Coleman spectrophotometer and the microdensitometer. In each instrument the data illustrated the same linear relationship between concentration and light absorption as for the other suspensions when plotted on semilogarithmetic paper. In Figure 10 can be seen the results obtained when the photronreflectometer and the Van Allen throm-

bocytocrits were used. In both instances, there is a linear relationship between concentration and instrument reading. These readings on the photronreflectometer are reported only for low concentrations of precipitate, since the graphs again flattened out at higher concentrations, as illustrated previously for both the red suspension and the lead chromate in Figures 5 and 8. It is to be noted that, when the white precipitate of aluminum hydroxide was used, the results, when

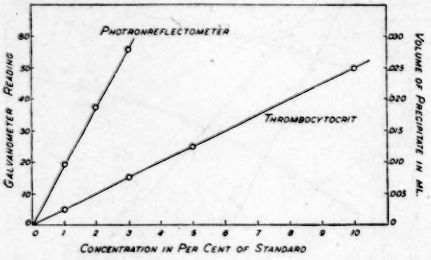

FIG. 10.—Data obtained from the photronreflectometer and Van Allen thrombocytocrits, using the same suspensions of aluminum hydroxide. Galvanometer readings at the left and volume of precipitate at the right are shown, respectively, for the photronreflectometer and the thrombocytocrits as obtained for various concentrations of precipitate.

graphed, are linear up to the maximum reference reading of 60 on the galvanometer scale used as the sensitivity setting.

In serological work the presence of hemoglobin in an antiserum in various amounts, on account of unavoidable contamination during preparation, may alter the readings obtained by transmittancy studies. With this in mind, a standard solution of oxyhemoglobin in Na_2CO_3 was prepared, and readings were made on the Coleman instrument and on the microdensitometer, the only two instruments of those used which measure the transmittancy of light by the test sample. That the effect of hemoglobin is considerable can be seen from the data plotted in Figures 11 and 12. It can also be seen that the region of the spectrum where the greatest absorption occurs is around 400 mμ, the same region where, owing to the fact that the higher wave lengths are less absorbed, studies on turbidimetry should be made. However, the use of proper controls eliminates this possible disturbing factor.

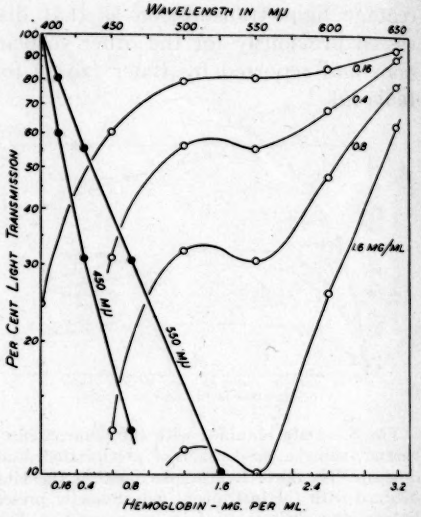

FIG. 11.—Data of Coleman spectrophotometer, using a solution of ox hemoglobin. The solid-circle graphs illustrate the relationship between concentration and transmission for two different wave lengths of incident light. The open-circle graphs show the data of variations in light transmission and wave length of incident light for four different concentrations of hemoglobin.

Finally, with due consideration to those factors which influence the instruments as shown by the previous studies, a serological study using the photronreflectometer and the microdensitometer was carried out and is illustrated by the data presented, respectively, in Figures 13 and 14. An anti-ox serum, diluted 1 : 6, was reacted against serial dilutions of the homologous ox-serum antigen, using dilutions of antigen to cover the range of precipitate formation. One

measure of the degree of reaction occurring is obtained by the process of summation of ordinates for each curve. This value, proportionate to the area under the curve, is assigned to the homologous reaction as equal to 100 per cent. In a study of animal relationships, the same antiserum would be reacted against heterologous antigens, and the summation of ordinates of the heterologous reaction curves would be compared to the value obtained for the homologous curve on a percentage basis.

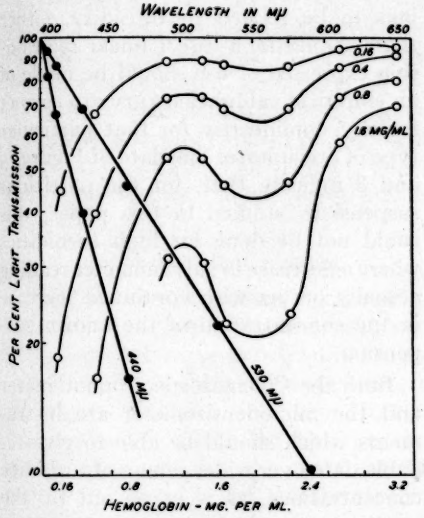

FIG. 12.—Data of microdensitometer, using a solution of ox hemoglobin. The solid-circle graphs illustrate the relationship between concentration and transmission for two different wave lengths of incident light. The open-circle graphs show the data of variations in light transmission and wave length of incident light for four different concentrations of hemoglobin.

DISCUSSION

The data presented in the previous section of this paper were obtained by using certain procedures. The direct-volume measurement studies have followed, without any change, the method outlined originally by Boyden and Baier (1929) and further by Baier (1933) and used in a relationship study by Baier and Wolfe (1942). The data of Figure 10 in this paper further support its use as a reliable technique for the measurement of precipitate, since a linear relationship exists between suspension concentration and thrombocytocrit reading up to the capacity of the calibrated capillary tube.

The use of the photronreflectometer has been reported on several occasions.

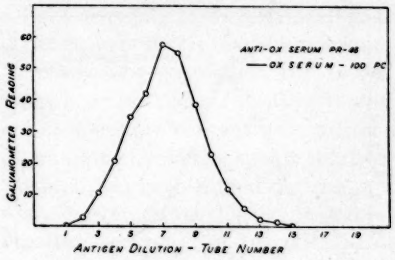

FIG. 13.—Application of the photronreflectometer to a study in serology.

It has been used by Bukantz, Cooper, and Bullowa (1941) for the study of turbidities resulting from the precipitation of solutions of pneumococcal III capsular polysaccharide (SSS) with pneumococcus III antipneumococcal serum. The unknown was diluted so that readings were made with the galvanometer deflections falling between 18 and 22 units on the instrument scale and with the reference light adjusted for an initial galvanometer reading of 15. Martin (1943) used the photronreflectometer in precipitin titrations of a pneumococcus type I polysaccharide (SSS) with antipneumococcus type I rabbit serum. The data presented in his paper were supported by the use of the standard deviation, indicating the precision of the instrument in being able to duplicate data. Roepke and Fitch (1940) report that they did not find the

photronreflectometer giving comparable readings to volumetric and antigen sensitivity studies on *Brucella abortus* suspensions. The instrument has been successfully used by Boyden (1939, 1942, 1943), Boyden and DeFalco (1943), and DeFalco (1941, 1942) in the study of animal relationships within the Crustacea, Aves, and Mammalia.

The data on instrument performance for this paper were obtained by the use of inorganic suspensions and were chosen for these studies because of their greater

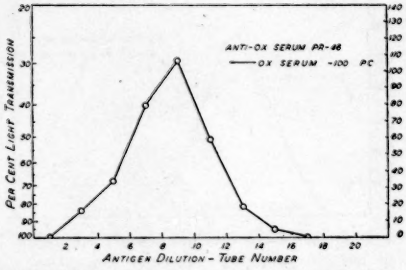

FIG. 14.—Application of the microdensitometer to a study in serology.

stability over the organic precipitates met with in serology. It is not intended to preclude the possibility that the instruments may behave differently toward serological precipitates. Such studies are in progress. However, on the basis of the studies reported in this paper, it should be pointed out, as a general precaution in the use of the photronelectrometer, that the practice of lowering the instrument sensitivity, so as to measure higher turbidities, should be carried out only to the extent that galvanometer readings are in direct relationship to turbidity concentrations for the particular instrument used and for the particular type of suspension system being studied.

The use of the standard error or standard deviation can indicate the precision of the instrument in reproducing data for any given set of reactants. The use of a series of known suspension concentrations of the particular type being studied will show whether the instrument exhibits a linear relationship between galvanometer reading and concentration. If the instrument is to be used to measure a reaction in terms of the amount of turbidity formed, then, for instrument readings to be related to turbidity, either there should be a direct linear relationship expressed or use should be made of an empirical calibration curve to correct for any nonlinearity for that particular type of precipitate. The data of Figures 5 and 8 indicate that, for the particular suspensions studied in this paper, this could not be done for high turbidities where a decrease in galvanometer reading actually occurs with continued increase in the concentration of the known suspension.

Both the Coleman spectrophotometer and the microdensitometer are instruments which should be able to give reliable data over wider ranges of turbidity concentrations, as is borne out by the studies reported in this paper, as well as by much additional unpublished data of the writer. However, these instruments are not nearly so sensitive to variations in degree of turbidity, nor can they detect turbidity to as low a concentration as the photronreflectometer.

Finally, the serological data reported in this paper indicate that, if due consideration in practice is given to the principles and theory of operation of both the photronreflectometer and the microdensitometer, the results obtained from both instruments are of comparable value and agree with data obtained by direct measurements of precipitate volume when Van Allen thrombocytocrits were used.

PROCEDURES FOR USE OF PHOTOELECTRIC INSTRUMENTS IN SEROLOGY

Since the instruments will be used to measure the light that is scattered or absorbed by suspended particles produced in the reaction mixtures composed of known amounts of antiserum and antigen, controls must be employed to make allowance for coloring matter and suspended matter naturally present in the antiserum and antigen. This must be done before the readings from the final mixtures are obtained. Procedures for the use of photoelectric instruments applied to studies in serology, based on the theory of their operation and taking into account the studies in this paper are as follows:

A. PHOTRONREFLECTOMETER

The instrument operates on the principle (1) that two collimated beams of light (from the same source and of regulated intensity), one a reference beam to the edge of the light-sensitive surface of the photocell, the other striking an opaque disk covering the center of the photocell, are adjusted in intensity so that the reference beam causes a maximum galvanometer reference reading (usually 60 scale units) without any absorption cell in the apparatus to block this reference light beam; and (2) that, when the absorption cell containing the control sample is in place in the receptacle, it will interrupt the adjusted reference light beam to the photocell but will allow the second beam to pass through the cell containing the control sample and to strike the opaque disk, and under these conditions the galvanometer will register approximately zero, and by means of the zero adjustment on the galvanometer an exact zero setting can be made so as to correct for inherent scattering of light by the control sample in the absorption cell; and (3) that with the unknown suspension replacing the control sample in the absorption cell and placed in the instrument, the presence of turbid matter in the unknown will cause a scattering of light to the photocell surface, resulting in a reading on the galvanometer proportionate to the degree of turbid material. Therefore, the procedure used in this paper is as follows:

a) With the galvanometer originally set at zero by means of the zero adjustment, turn on the light and adjust the light intensity to the photocell so that the galvanometer reads maximum (60 units). The absorption cell is *not* in the instrument for this adjustment.

b) Fill a sample cell with 1.7 ml. of 0.9 per cent physiological salt solution to which has been added 0.3 ml. of the antiserum, either diluted or undiluted as the case may be. Insert this cell in the instrument and reset the galvanometer zero adjustment so that the galvanometer reads zero.

c) Remove the cell and readjust the maximum setting as under *a* above, and repeat *b*, so that zero and maximum settings are correctly made. This is the antiserum and diluent control adjustment for any inherent suspended matter in these solutions.

d) Prepare a number of serial dilutions of the antigen in the same physiological salt solution as used for *b*, and transfer 1.7 ml. to each of a series of similar absorption cells. Read these in the instrument for inherent suspended matter. These readings are recorded separately for each antigen dilution as the control readings for the respective antigen dilutions.

e) To each antigen dilution cell of *d* add 0.3 ml. of antiserum of the same dilution as used in *b* above. After incuba-

tion for 20 minutes at 37.5° C., record the galvanometer readings for each absorption cell. The antiserum control of *b* is available and was incubated along with the reaction cells, for rechecking the zero setting.

f) The final reading corresponding to the degree of turbidity in the reaction of antiserum and antigen for each dilution of antigen is the galvanometer reading of that antigen dilution obtained under *c minus* the control reading obtained under *d* for that antigen dilution. These readings are plotted as indicated in Figure 13. If any of the readings under *e* exceed the original maximum setting as under *a* the procedure may be modified by using a maximum setting of the galvanometer less than full scale, multiplying all galvanometer deflections by a factor dependent upon the new maximum setting chosen. The degree to which this procedure is followed is dependent upon the extent to which light-scattering measurements are known to be linear for the particular instrument and type of suspension being studied. Also, a sufficient number of antigen dilutions must be made so that in both the lowest and the highest antigen dilution cells the final readings are zero, indicating that the complete antigen-antibody precipitation range has been covered.

The time and temperature of incubation, as well as the volume and the concentration of the physiological saline and reactants, can be varied, depending on the nature of the study under investigation.

B. MICRODENSITOMETER

This instrument measures the total transmission of the sample, in percentages when compared to the control as transmitting 100 per cent. The principles of operation have been fully described by Baier (1943). The procedure in using the instrument is as follows:

a) Prepare two 75 × 10-mm. serological test tubes containing 1 ml. of physiological saline, and set the instrument at the null-point, with both tubes in position in the light beams going to each of the two photocells in the bridge circuit and with the transmittance scale set at 100. This is the control adjustment for the diluent.

b) Prepare serial antigen dilutions. Transfer 1 ml. of each dilution to each of a series of 75 × 10-mm. tubes. Read the transmittancy of each antigen dilution in the instrument. These readings subtracted from 100 give the absorption values in percentages for each antigen dilution and will be used as the respective antigen controls.

c) To each antigen dilution of *b* add 0.3 ml. of antiserum. Incubate the mixtures for 20 minutes at 37.5° C.

d) While these tubes are incubating, prepare two 75 × 10-mm. serological test tubes containing 1 ml. of saline and 0.3 ml. of antiserum diluted as in *c*. Place them in the instrument as in *a*. Adjust the instrument for the null-setting, with the scale at 100 per cent transmittancy, as in *a*. This corrects for the absorption of the antiserum and diluent and adjusts the instrument to be ready for the readings to be made under *e*.

e) After incubation of the antigen-antiserum mixture, as prepared in *c*, read each tube in the instrument against the antiserum and diluent control, as prepared in *d*.

f) The final reading, corresponding to the degree of turbidity in the reaction of antiserum and antigen for each di-

lution of antigen and to be used in plotting the graphs in determining relationship values, is obtained by using the reading for that antigen dilution as obtained under *e* and subtracting from each the reading obtained in *b* for that antigen dilution. Again, antigen dilutions must be chosen so that the final readings for the highest and lowest dilutions will intercept the zero axis of the graph.

tube was used in preparing the graph and the table. This number of dilutions was sufficient to give reliable results as determined from a number of such studies. The value to be used to represent the total reaction could be obtained by determining the summation of the ordinates of the graph. In a relationship study the summation of the ordinates of the graphs for the heterologous reactions would be determined in the same way and would

TABLE 1

DATA OF THE PHOTRONREFLECTOMETER FOR AN OX : ANTI-OX PRECIPITIN STUDY

	Antigen Tube No.							
	1	3	5	7	9	11	13	15
Antigen dilution	12.5	62.5	250	1,000	4,000	16,000	64,000	256,000
Antigen control	0	0	0	0	0	0	0	0
Antigen-antibody precipitate reading	0.2	10.9	34.2	57.0	38.4	11.5	2.2	0
Reading *minus* antigen control	0.2	10.9	34.2	57.0	38.4	11.5	2.2	0

TABLE 2

DATA OF THE MICRODENSITOMETER FOR AN OX : ANTI-OX PRECIPITIN STUDY

	Antigen Tube No.								
	1	3	5	7	9	11	13	15	17
Antigen dilution	1	4	16	64	256	1,024	4,096	16,384	65,536
Antigen control	38.0	69.7	87.7	95.5	98.2	99	99.4	100	100
Reading of antigen-antibody mixture	38.0	53.4	55.5	39.8	27.7	49.5	80.7	95.0	100
Corrected reading	100	83.7	67.8	44.3	29.5	50.5	81.3	95.0	100

Variations in time and temperature of incubation, as well as concentration and volume of reactants, can be made as described previously. Light filters with a transmittancy peak of approximately 415 mμ are recommended.

ANALYSIS OF SEROLOGICAL DATA

Table 1 (abbreviated) illustrates the data of the photronreflectometer as plotted in Figure 13 for the reaction of anti-ox serum with ox antigen. To conserve space, only every other antigen dilution

be recorded in percentages of the value of the homologous value referred to as 100 per cent.

Table 2 illustrates the same procedure for the microdensitometer, with the same antiserum reacted against the same antigen, as plotted in Figure 14.

SUMMARY

1. The theoretical aspects of photometry applied to turbid systems are discussed in relation to their use in serology.

2. The relationship between turbidity and (1) transmitted light or (2) scattered light is presented for two types of photoelectric instruments, with suspensions of several different precipitates.

3. With suspensions up to a certain maximum concentration, there is expressed a linearity between concentration and scattered light measurement.

4. In light-transmission studies, a linear relationship exists between turbidity and the inverse logarithm of the transmission over a wider range of concentrations.

5. Procedures are suggested for the use of photoelectric instruments in serology.

6. A study in serology is presented, showing the parallelism of data from determinations of (1) scattered light measurements and (2) transmitted light measurements of the precipitate formed in reacting an antiserum against its homologous antigen.

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THE INFLUENCE OF THE SINUS GLANDS UPON MOLTING AND ASSOCIATED CHANGES IN THE CRAYFISH[1]

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THE crayfish and other crustaceans grow discontinuously by means of a series of periodic molts of their hard exoskeleton. There is considerable evidence to suggest that this molting process in crustaceans is hormonally regulated by the sinus glands of the eyestalks. The purpose of this investigation is to determine in greater detail the manner in which the sinus glands control molting and certain changes associated with the molting process in the crayfish, namely, gastrolith formation, changes in the exoskeleton, water metabolism, growth, and oxidative metabolism. The normal physiology of molting was considered, first, and then the role of the sinus glands in the regulation of molting was determined by comparing changes in normal, eyestalkless or sinus-glandless, and eyestalkless crayfish with sinus-gland implants or injections of sinus-gland extracts. Most of the results to be presented have been summarized in abstracts previously reported (Scudamore, 1941, 1942*a*, *b*).

Molting in crustaceans is a complex process which involves a series of associated changes progressing over a period of several days. For example, Baumberger and Olmsted (1928) and Baumberger and Dill (1928) have shown that a series of changes in water content, osmotic pressure, glycogen, and sugar content occur during the molt cycle of certain crabs. Drilhon (1933) and Drach (1939) also found variations in blood sugar during the molt cycle.

Definite changes in calcium metabolism are associated with molting. For several days preceding molt, calcium is resorbed from the exoskeleton and possibly other sources and stored in gastroliths, as in crayfish (Huxley, 1906; Robertson, 1941), or in the hepatopancreas, as in certain crabs (Paul and Sharpe, 1916; Robertson, 1937). However, different results were obtained by Maluf (1940), who reported that only about 4 per cent of the raw ash (mostly calcium) and 2.3 per cent of the organic material of the old cuticle is resorbed in crayfish, and Kleinholz and Bourquin (1941*b*), who found no significant storage of calcium in *Uca* before molting. Following shedding of the exuvium, the new cuticle is hardened partly by redeposition of the stored calcium but primarily by addition of freshly acquired calcium from the environment (Hecht, 1914; Paul and Sharpe, 1916; Maluf 1940; Kleinholz and Bourquin, 1941*b*). Maluf (1940) stated that the storage of calcium in the gastroliths of the crayfish is of little significance in calcifying the cuticle after molt, but that Ca^{++} ions, absorbed from the environmental water directly through the integument, are the chief source of calcium. Increases in the blood calcium during the formation and dissolution of the gastroliths in *Sesarma* were reported by Numanoi (1939).

[1] This investigation is a portion of a thesis submitted in partial fulfilment of the requirements for the Ph.D. degree in zoölogy at Northwestern University, in June, 1942. The author wishes to express his sincere appreciation to Dr. Frank A. Brown, Jr., for his inspiration and helpful criticisms during the course of this investigation.

There is also a cycle of changes in the water metabolism associated with molting. Increases in weight, chiefly due to increased water content, preceding or immediately following molt in several crustaceans were recorded by Paul and Sharpe (1916), Olmsted and Baumberger (1923), Robertson (1937), Drach (1939), Maluf (1940), and Kleinholz and Bourquin (1941*a*). Baumberger and Olmsted (1928) found that both osmotic pressure of body fluids and water content increased preceding molt in *Pachygrapsus* and decreased following molt.

Growth is an important result of molting. Immediate increase in weight due to increased water content has been mentioned, and there is a smaller but permanent increase in weight after molt. Van Deventer (1937) and Tack (1941) found that crayfish do not increase markedly in size at the spring molt but do increase 10–30 per cent in carapace length with the summer molt.

There is a paucity of reports of variations in oxygen consumption during the molt cycle. However, Helff (1928) found that there were no marked changes in oxygen consumption of eight crayfish that molted during the course of his experiments, which lasted only a few hours.

The seasonal life-history of *Cambarus immunis* and *C. propinquus*, as described by Van Deventer (1937) and Tack (1941) and observed by the author, consists essentially of a fall mating season, winter intermolt and period of inactivity, spring spawning season, spring molt, spring intermolt, and summer molt. The young of *C. immunis* hatch in early spring, undergo two molts while clinging to the pleopods of the female, and experience several additional molts during the summer. In the second and subsequent summers there is a spring molt period (mid-April and May), a spring intermolt period, and a summer molt period (mid-June to August), followed by a long winter intermolt period. The difficult shedding of the exoskeleton during the molting process has been adequately described by Huxley (1906) and others. Many crayfish die at the time of ecdysis, and very few live as long as 4 years; most crayfish die in the second or third years of life. According to Huxley (1906), the gastroliths begin to form 40 days before molt in 4-year-old *Astacus* but only 10 days before molt in first-year crayfish. Based upon these premolt periods, it is probable that gastroliths begin to form 20–30 days before molt in 1-, 2-, or 3-year-old crayfish.

With this introduction to the complexity of the molting process and the life-cycle of the crayfish, it should be possible to interpret the influences of the sinus glands in controlling molting and the associated changes.

Megušar (1912) was the first to report that ecdysis occurred at more frequent intervals in eyestalkless than in normal *Astacus*. This acceleration of molting following eyestalk removal was later observed in *Uca pugilator* by Abramowitz and Abramowitz (1938, 1940) and Kleinholz and Bourquin (1941*a*); in *C. immunis* by Brown and Cunningham (1939); in *Eriocheir sinensis* by Hanström (1939); and in young *C. clarkii* by Smith (1940).

Brown and Cunningham (1939) have shown that the acceleration of molting after eyestalk ablation probably was due to the removal of a molt-inhibiting hormone produced by the sinus glands, since implantation of sinus glands into eyestalkless crayfish definitely delayed the onset of molt. Further evidence for the hormonal inhibition of molt by the sinus glands was obtained, independently, by Kyer (1942) and Scudamore (1942*a* and this paper), who demonstrated that sinus-gland implants in eyestalkless crayfish definitely delayed the for-

mation of gastroliths, which otherwise developed after eyestalk extirpation. Recently, Pyle (1943) described cyclic changes in the histology of the sinus glands of *Cambarus* correlated with the molt cycle, in which acidophylic granules predominated prior to molt and mainly basophilic ones just after molt.

The mechanism of the initiation and acceleration of molt, either in normal or in eyestalkless animals, has not been fully explained. However, Plankemann (1935) suggested that starvation, increased metabolism, color of background, and pH of sea water might affect the rate of molting. Darby (1938) stated that operative injury hastened the onset of molt in *Crangon* (= *Crago*), although Smith (1940) showed that this was not true in young crayfish. The importance of temperature upon the rate of molting and gastrolith formation was revealed by Smith (1940), Hess (1941), and Kyer (1942). Kleinholz and Bourquin (1941*a*) reported that inanition delayed the onset of molt in eyestalkless *Uca*.

There have been some reports of hormonal regulation of some changes associated with molting. Humoral control of calcium metabolism was suggested by Koller (1930), who reported that the molted exoskeletons of eyestalkless *Crangon* (= *Crago*) *vulgaris* contained less calcium (actually salts soluble in dilute hydrochloric acid) than normal ones. Plankemann (1935) made similar observations on other crustaceans. However, Kleinholz and Bourquin (1941*b*) found no difference in the amount of calcium in the molted exoskeletons of normal and eyestalkless *Palaemonetes*. They also reported that the calcium content of the exoskeleton of eyestalkless *Uca* was only 58 per cent of normal, 30 days after molt, at which time they were molting again, although the authors did not compare the rate of calcium deposition with normal animals. By delaying the second molt by inanition, the calcium was increased to 86 per cent of normal.

An increased size of eyestalkless animals as compared to normal ones, due to increased frequency of molts, was described in *Uca* by Abramowitz and Abramowitz (1940) and Kleinholz and Bourquin (1941*a*) and in young crayfish by Smith (1940).

There has been some evidence to suggest that the sinus glands are necessary for life (viability-effect) because eyestalk removal definitely shortens the life of crustaceans (Brown, 1938, 1939, 1940; Brown and Cunningham, 1939; Smith, 1940; and Abramowitz and Abramowitz, 1940). Brown and his co-workers demonstrated that sinus-gland implants extended the life of eyestalkless animals. However, Herbst (1902), Megušar (1912), and possibly others presumably have kept eyestalkless animals alive over long periods of time. Kleinholz and Bourquin (1941*a*) doubt the existence of a viability-effect and suggest that mechanical factors during molting are largely responsible for the deaths of eyestalkless animals. Brown (1944), in a recent review of crustacean hormones, has discussed the viability problem in detail.

MATERIALS AND METHODS

The fresh-water crayfish *C. immunis* Hagen and a few *C. virilis* Hagen were used as experimental animals in this investigation. The animals were kept in lead-lined tanks filled with running water and were occasionally fed small amounts of earthworm, liver, and crayfish. During experiments the animals were placed either in individual glass finger bowls, which were filled and frequently replenished with fresh tap water maintained at room temperature (20° ± 2° C.), or in respirometers. Nei-

ther experimental nor control crayfish were fed during an experiment. It had been observed that eyestalkless crayfish invariably ate ravenously whenever food was offered, whereas normal animals often refused to eat.

The eyestalks, containing the sinus glands, were removed either by excising with a sharp scalpel and searing the wound with electric cautery or by ligating at the basal membrane with a silk thread and cutting distal to the ligature. In some experiments the sinus glands were removed by the technique described by Brown (1942), leaving the eyestalks intact without appreciable injury to the nervous tissue and ommatidia.

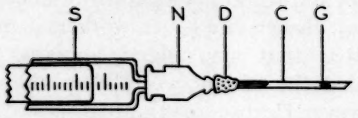

FIG. 1.—Device used in making sinus-gland implantations: *S*, tuberculin syringe; *N*, hypodermic needle; *D*, De Khotinsky cement; *C*, glass capillary tube; and *G*, sinus gland.

The technique used for implantation of viable sinus glands was one developed in our laboratories during the past few years. Eyestalks were removed and placed in a Syracuse watch glass containing a small quantity of the saline solution proposed by Van Harreveld (1936). With the aid of a strong beam of light and a dissecting microscope, the contents of the eyestalk were withdrawn from the open, proximal end with fine-pointed watchmaker's forceps, and the sinus gland was separated from its position between the medulla interna and the medulla externa. The device used for implantation (Fig. 1) consisted of a tuberculin syringe with a fine glass capillary tip attached to a 26-gauge hypodermic needle with De Khotinsky cement. It was possible to draw the sinus gland, with a small quantity of saline, into the capillary tip, where it was plainly visible. The implants were placed in the ventral abdominal tissues by making a small incision between two sternites of the abdomen, inserting the capillary tip about 5 mm., and injecting the implant into the tissues surrounded by the blood sinuses with the precision of the syringe plunger. Such implants retained their characteristic appearance and probably were viable for 1–3 weeks.

Sinus-gland extracts were prepared by dissecting the sinus glands from the stalk tissue as described above, placing them in a small mortar (tip of Pyrex test tube), allowing them to dry, and then triturating the dried glands with a measured amount of Van Harreveld's solution. The tube with the extract was placed in a beaker of boiling water, cooled, and either filtered or drawn immediately into a 1-cc. tuberculin syringe equipped with a 26-gauge needle. Extracts of nervous tissue or muscle were prepared by a similar method. The injections of 0.05 cc. of such an extract were made into blood sinuses through the membrane at the base of a walking leg.

The carapace length was determined by measuring, with a vernier caliper, the straight distance to tenths of a millimeter from the tip of the rostrum to the posterior margin of the carapace. The gastroliths were dissected from the anterior wall of the stomach, dried in an oven at 100° C. for 24 hours, and the weight determined to tenths of a milligram. To determine weights and weight changes, the animals were dried with several pieces of absorbent tissue and weighed rapidly. Slight errors in weights due to small amounts of water trapped in the gill chambers or to evaporation were possible in this method. The water content was determined by sacrificing an animal after weighing, drying in an oven

at 100° C. for 24 hours, and re-weighing to determine the dry weight. The difference represented the fluid or water content.

The calcium content (actually, salts soluble in dilute hydrochloric acid) of the exoskeleton was determined by sacrificing a crayfish, removing the carapace, scraping the hypodermis from the cuticle with a scalpel, and analyzing the soluble salt content. The carapace was dried, weighed on an analytical balance, soaked in 2 N hydrochloric acid for 12 hours to remove soluble salts, washed in distilled water, dried, and re-weighed. The difference in weights gave a measure of the soluble compounds contained in the cuticle, and the results were expressed as percentage of soluble materials on a basis of dry weight. Since the soluble compounds are primarily calcium, this method gives a rough measure of the calcium changes.

The rate of oxygen consumption was determined by placing the crayfish in respirometers filled with tap water and kept in a constant-temperature bath, which was maintained at 20° ± 0.1° C. throughout the experiments. One calibrated sample bottle was filled with the supply water to determine the initial oxygen concentration, and another sample was siphoned off from each respirometer at the end of the experiment (60 minutes) to ascertain the final oxygen concentration. The respirometer consisted of a 500-cc. Erlenmeyer flask fitted with siphon tubes for filling and emptying without modification in oxygen content. Crayfish of 21–31 mm. carapace length and 2.0–8.0 gm. body-weight were used so that the oxygen concentration in the respirometer remained within the range at which the rate of oxygen consumption was independent of oxygen tension, as shown by Helff (1928), Hiestand (1931), Maluf (1936), and others. The tap water was aërated and adjusted to 20° C. before an experiment was begun, and the crayfish were placed in partly filled flasks in the constant-temperature bath for 3 days prior to an experiment to adjust them to the respirometers and to the 20° C. temperature. The oxygen concentration was determined by a modification of the Winkler method, as recommended by the American Public Health Association (1933). The 0.01 N sodium thiosulphate used in the titration was standardized weekly against a standard solution of potassium dichromate. The concentration of the dissolved oxygen was calculated as cubic centimeters of oxygen per liter, and the results were expressed as cubic centimeters of oxygen consumed per hour. The results were probably accurate within 0.005 cc. of oxygen per liter. Preliminary tests, using the precautions recommended by Allee and Oesting (1934), proved the method to be satisfactory. Later experiments in our laboratory with the Van Slyke manometric apparatus have verified the results obtained by the Winkler method.

MOLTING AND GASTROLITH FORMATION

Experiments were performed during the winter of 1941–42 to study the role of the sinus glands in the regulation of gastrolith formation preceding ecdysis in the crayfish, because, in nature, winter is a long intermolt period.

EXTIRPATION EXPERIMENTS

Several crayfish were divided into three groups and placed in individual finger bowls filled with water at room temperature. In one group both eyestalks were removed from each animal; in a second group a single eyestalk was removed from each one; and in a third group there was no operation. As the eyestalkless animals died, the gastroliths were re-

moved and weighed. Periodically, the normal control animals and animals with a single eyestalk removed were also sacrificed and examined for the presence of gastroliths. The results are summarized in Table 1.

TABLE 1

EFFECTS OF EYESTALK REMOVAL ON GASTROLITH FORMATION AND MOLTING IN CRAYFISH (OCTOBER–NOVEMBER)

	Bilateral Eyestalk Removal	Unilateral Eyestalk Removal	Control Animals
Number examined	21	13	19
Gastroliths present:			
After 4–27 days	21	0	1
After 37–91 days		3	1
Number molted	5	1	1
Average gastrolith weight (mg.)	19.87	3.50	1.99
Range in carapace length (mm.)	21–27	20–25	20–27

Gastroliths were present in all the eyestalkless crayfish examined during the first 27 days of this experiment, and five of the crayfish molted during this period of time. Only two of the control animals developed gastroliths, and one of these molted (twentieth and forty-sixth days of observation). None of the crayfish with a single eyestalk removed developed gastroliths within 27 days, although three animals (44, 63, and 73 days after operation) contained small gastroliths, and one of them molted. One eyestalk (sinus gland) seemed adequate to inhibit molt.

Eyestalkless crayfish that molted and survived were observed to determine whether they would immediately enter into a second molt, as indicated by the formation of a second set of gastroliths. Crayfish that lived only a week or less still had the old gastroliths in the stomach, incompletely dissolved. However, all nine eyestalkless crayfish, which were examined at death 2–3 weeks after the previous molt, contained a second set of gastroliths (Table 2). Animal 1 appeared ready to molt, as evidenced by a loosened cuticle, although the gastroliths were only about one-fourth their usual size at the time of molt. In most instances several days would have been required for another molt to take place, making the second premolt period about twice the first. In all cases the eyestalkless crayfish seemed to be approaching a second molt immediately after the first.

A final experiment was performed to determine whether the sinus gland was the factor in the eyestalks, whose removal resulted in gastrolith formation and molting. Both sinus glands were removed from one group of crayfish; both eyestalks from a second group; and normal animals were used as a control group. One animal from each group was sacrificed at intervals and examined for gastroliths. The results are summarized in

TABLE 2

GASTROLITH FORMATION IN EYESTALKLESS CRAYFISH WITH A 23.8-MM. AVERAGE CARAPACE LENGTH APPROACHING A SECOND MOLT (OCTOBER–DECEMBER)

Animal No.	Days after First Molt	First Premolt Period (Days)	Weight of Pair of Gastroliths (Mg.)
1	16	13	15.4
2	19	15	0.5
3	19	22	Trace
4	20	17	0.5
5	21	15	0.8
6	23	14	11.2
7	23	19	1.4
8	25	16	5.9
9	27	16	1.0

Table 3. Bilateral sinus-gland extirpation resulted in gastrolith formation in all cases, although at a slightly slower rate than after bilateral eyestalk ablation. The interpretation was complicated by the fact that normal animals, which had

been in the laboratory for some time, were beginning to form gastroliths for the spring molt. Post-mortem examination of the eyestalks revealed that the stalk tissue was not seriously injured and that no sinus-gland tissue or only tiny fragments remained. One sinus-glandless animal molted 20 days after operation. This experiment indicates that sinus-gland removal is sufficient to initiate gastrolith formation and molting.

REPLACEMENT EXPERIMENTS

Additional experiments were performed to determine whether sinus-gland implants would prevent the gastrolith formation and molting which followed eyestalk (sinus-gland) removal. Bilateral eyestalk extirpations were performed on fifty-four crayfish, which were then divided into two equal groups. The first group received a sinus-gland implant every 3 or 4 days until two to six had been made, and the second group received implants of eyestalk tissue from

TABLE 3

GASTROLITH FORMATION IN GRAYFISH OF 21–26-MM. CARAPACE LENGTH (FEBRUARY–MARCH)

Days after Operation	Weight of Gastroliths (Mg.)		
	Normal	Sinus-gland-less	Eyestalk-less
2	0	Trace	Trace
4	0	0.2	0.8
7	0.6	2.6	1.9
10	0.8	2.1	10.7
11	0	12.6	23.1
13	Trace	.....	25.5
17	Trace	8.2	35.2
20	0	Molt	Molt

which the sinus glands had been removed. The crayfish were sacrificed at frequent intervals to examine for the presence of gastroliths.

The implantation of sinus glands prevented or retarded the formation of gastroliths and, as long as fresh implants were added, prevented molting (Table 4). Implants of eyestalk tissue without the sinus gland were unable to prevent gastrolith formation and molting. All these animals had molted or died with

TABLE 4

EFFECT OF SINUS-GLAND IMPLANTS (ONE TO SIX) IN EYESTALKLESS CRAYFISH ON GASTROLITH FORMATION AND MOLTING (OCTOBER–JANUARY)

	Stalk-Tissue Implants	Sinus-Gland Implants
Number examined	27	19
Gastroliths present in	25	9
Number molted	9	0
Duration of experiment (days)	22	37
Average gastrolith weight (mg.)	31.20	0.65
Range in carapace length (mm.)	22–26	21–25

large gastroliths in a period of 12–22 days, whereas the animals with sinus-gland implants had developed only small gastroliths after 37 days. Eight of the eyestalkless animals with sinus-gland implants were permitted to live for some time after the last implant. As shown in Table 5, all these animals eventually developed large gastroliths, and some of them molted. In all but two instances the period after the last implant was about twice as long as the premolt period in eyestalkless animals without sinus-gland implants. Therefore, the sinus-gland implants must have been effective for at least 7–10 days after implantation. Microscopic examination revealed that the sinus-gland implants retained their normal appearance and were apparently viable tissue. Two or three implants, given at 3–4-day intervals, seemed to be just as effective as six in preventing gastrolith formation and molting but probably depended on the viability of the implants.

Preliminary experiments demonstrated that daily injections of eyestalk or sinus-gland extracts were not so effective in preventing gastrolith formation as were the implants. Tests on a few animals suggested that daily injections of extracts of "brain" and thoracic ganglia increased the rate of gastrolith formation. The results merely suggested the possibility of a molt-accelerating substance in the central-nervous-system secretory cells.

TABLE 5

GASTROLITH FORMATION IN EYESTALKLESS CRAYFISH FOLLOWING THE LAST SINUS-GLAND IMPLANT (OCTOBER–JANUARY)

Animal No.	No. of Times Implanted	Days after Last Implant	Days after Eyestalk Removal	Weight of Gastroliths (Mg.)
1	6	15	37	Molt
2	6	17	39	42.4
3	6	25	57	13.0
4	6	26	48	8.7
5	4	27	42	Molt
6	3	27	42	Molt
7	2	29	35	29.7
8	3	32	42	29.1

FREQUENCY OF MOLTS

A summary of molting that occurred among crayfish observed during the winter of 1941–42 is listed in Table 6. The highest percentage of molts was noted in the eyestalkless crayfish (41.38 per cent). An even higher percentage of molts would have been recorded except for the early death of the rest of the eyestalkless animals, because all that died had formed gastroliths. Eyestalk tissue, without the sinus glands, implanted into eyestalkless animals did not prevent the high frequency of molts (33.33 per cent). However, sinus-gland implants prevented molting in eyestalkless animals as long as viable implants were present. But large gastroliths formed in some, and others molted 15–32 days after the last implant. The few cases of molting in normal crayfish and in those with one eyestalk removed were all small animals (yearlings), and probably represented either delayed summer molts or the effect of the laboratory temperatures. The average length of premolt period (eyestalk removal to ecdysis) in the eyestalkless crayfish was 16.26 ± 0.68 days,[2] but varied from 11 to 22 days. This interval was much shorter than the normal spring and summer premolt periods of 20–30 days, as based on notes of Huxley (1906). Furthermore, winter is normally a long intermolt period devoid of molting, which adds further significance to this report of gastrolith formation and molting after eyestalk or sinus-gland removal.

RATE AND RHYTHM OF GASTROLITH FORMATION

Since animals were sacrificed every few days to examine for gastroliths, it was possible to study the rate of gastrolith formation. A graph showing the distribution of gastrolith size with time after eyestalk removal is shown in Figure 2, curve *A*. Animals killed from 2 to 8 or 10 days after eyestalk removal contained

TABLE 6

SUMMARY OF MOLTING IN CRAYFISH OBSERVED DURING THE WINTER OF 1941–42

Experimental Condition	No. of Animals Observed	No. of Animals Molted	Percentage of Animals Molted
Normal	600	5	0.83
One eye off	13	1	7.69
Two eyes off	58	24	41.38
Two eyes off, sinus-gland implants	27	0*	0.00
Two eyes off, stalk-tissue implants	27	9	33.33

* Molting did occur in some, 15–32 days after last implant.

[2] The standard error of the mean is used in all statistical computations unless otherwise indicated.

very small gastroliths. In the next few days the gastroliths became much larger, and the animals molted (Molt I) in from 11 to 22 days postoperatively. There was considerable individual variation in gastrolith size. Crayfish that molted and lived longer than 1 week had already developed another set of small gastroliths (Molt II) in preparation for a second molt. In contrast, eyestalkless crayfish with sinus-gland implants and normal animals contained no, or very small, gastroliths when examined during the same time intervals (Fig. 2, curve *B*). In a second experiment eyestalkless animals of the same size range were kept under uniform conditions, and an animal was sacrificed and examined for gastroliths at regular intervals. The results are shown in Figure 3. Unfortunately, all the experimental animals were killed before any had molted. However, in both experiments there was no appreciable increase in gastrolith size during the first 7 days after eyestalk removal and then slow growth for 2 or 3 days, followed by

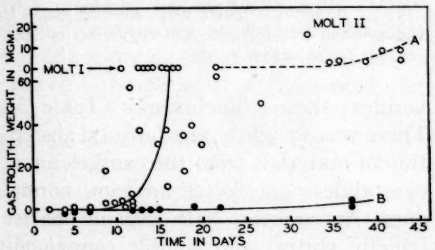

FIG. 2.—Rate of gastrolith formation in the winter in crayfish (23.5–27.5 mm. carapace length) following eyestalk removal (curve *A*) as compared to normal crayfish or eyestalkless ones with sinus-gland implants (curve *B*). Molt I indicates approximate gastrolith size at the time of molt, and Molt II indicates gastroliths forming for a second molt.

rapid growth for about a week before ecdysis. These changes in size are clearly indicated in Figure 4, *B*.

When gastroliths were sectioned with a sharp scalpel and examined under a dissecting microscope using reflected light, they were found to consist of a series of concentric layers of materials (Fig. 4, *A*). Small gastroliths had a few layers, and larger ones had more layers. The largest gastroliths from eyestalkless crayfish at

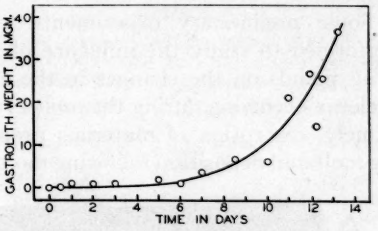

FIG. 3.—Rate of gastrolith formation in individual crayfish (27.2 mm. average carapace length) following eyestalk removal, under uniform environmental conditions.

or near the time of molt consisted of seven or eight narrow layers, against a thin, chitin-like disk, surrounded by five to nine thick layers, which were thickest farthest from the chitin-like disk. Since the number of layers corresponded closely with the number of postoperative days, the possibility of a daily rhythm in deposition of the lime salts was suggested. To test this hypothesis, both eyestalks were extirpated from several crayfish at 5:00 P.M., and thereafter an animal was sacrificed each day, and the layers in the gastroliths were determined. As shown in Table 7, the number of layers in the gastroliths corresponded perfectly with the number of days after eyestalk removal. Examinations in the morning and afternoon suggested that the layers were deposited nocturnally. A second experiment, started at 8:00 A.M., revealed essentially the same results. A final experiment was started at 11:00 A.M., gastroliths were permitted to develop for 8 days, and an animal was sacrificed and the gastroliths examined every 3 hours for a 24-hour period. The deposition of a new layer had started by

4:00 P.M. and was completed before noon of the following day. This evidence seemed to suggest a daily rhythm in gastrolith formation, with the greatest activity in deposition occurring at night.

CHANGES IN THE EXOSKELETON

Some preliminary experiments were performed to study the influence of the sinus glands on the changes in the exoskeleton occurring during the molt cycle, namely, resorption of materials preceding molt and deposition following molt.

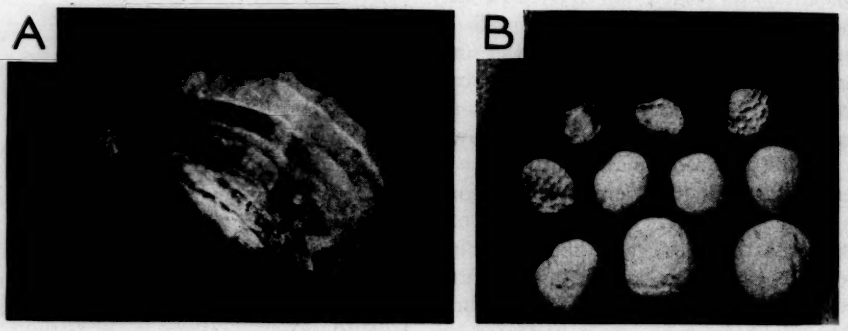

FIG. 4.—*A*, cross-section of a gastrolith 14 days after eyestalk removal, indicating seven thick outer concentric layers of deposited materials (enlarged *ca.* 10×). *B*, series of gastroliths, showing stages in the increase in size. Starting with upper row and reading from left to right, the days after eyestalk removal for each gastrolith are: 5, 7, 8, 10, 12, 12, 11, 12, 13, 14 (enlarged *ca.* 3×).

The percentage of soluble materials (chiefly calcium compounds) in the carapace was determined in crayfish under various experimental conditions and at different stages in the molt cycle, as shown in Table 8. The intermolt carapace contained a lower percentage of soluble materials (72 per cent) than did the molted carapace (78.5 per cent) from normal spring molts. A few days before molt the carapace of normal animals contained an intermediate amount of soluble materials (76.9 per cent). The percentage of soluble compounds in the molted carapace of eyestalkless crayfish (79.5 per cent) in the winter was essentially the same as that found in the molted carapace of normal animals at the spring molt.

Further observations, as listed in Table 8, as well as other nontabulated results, indicated that materials were removed from the exoskeleton, because the total dry weights of the molted carapaces were less than those of intermolt carapaces of the same length. The carapace of animals about to molt also weighed less than comparable intermolt carapaces. The comparison of the ratios of total dry weight (mg.) divided by length (mm.) verifies these conclusions (Table 8). There was a slightly greater total absorption of materials from the exoskeleton of eyestalkless crayfish than from normal ones. Apparently, both organic matter (chiefly chitin) and soluble compounds (mainly calcium) were removed from the cuticle during molting, the organic matter being absorbed proportionately faster, as indicated by the increase in percentage of soluble materials in the molted carapace.

The rate of deposition of materials in the new cuticle following ecdysis was studied by sacrificing molted animals at intervals after molt and testing the carapace for soluble materials. As illustrated

in Figure 5, the percentage of soluble materials in the exoskeleton (and the total carapace weight) increased rapidly for the first 4 or 5 days and then increased at a slower rate during the time observed. However, the winter intermolt carapace percentage composition and total weight never were attained in the period studied. The early rapid increase probably represented solution of the gastroliths and deposition of this material in the cuticle, while the slow increase represented addition from environmental sources. One crayfish, not included in the results, devoured part of its molted exoskeleton and had a much higher proportion of soluble materials in its carapace when examined. The percentage composition of the carapace in normal and eyestalkless crayfish was essentially the same (Fig. 5, *A*). However, the total amount of soluble materials deposited seemed to proceed at a slower rate in eyestalkless, than in normal, animals, as was shown by comparing the ratio of weight of soluble compounds (mg.) divided by carapace length (cm.) in Figure 5, *N–E*. These findings suggest that the eyestalk sinus glands may be essential to complete the process of hardening the exoskeleton by the secretion of chitin and the deposition of lime salts.

TABLE 7

TIME OF DAY AT WHICH THE LAYERS OF CALCIUM COMPOUNDS ARE DEPOSITED IN THE GASTROLITHS OF EYESTALKLESS ANIMALS

Animal No.	Time Examined	Days after Eyestalk Removal	No. of Layers Present in Gastroliths	No. of Layers, if Deposited Diurnally	No. of Layers, if Deposited Nocturnally
1.....	5:00 P.M.	1	1	1	1
2.....	5:00 P.M.	2	2	2	2
3.....	9:00 A.M.	2½	3	2	3
4.....	5:00 P.M.	4	4	4	4
5.....	5:00 P.M.	6	6	6	6
6.....	9:00 A.M.	7½	8	7	8
7.....	5:00 P.M.	8	8	8	8
8.....	9:00 A.M.	8½	9	8	9
9.....	5:00 P.M.	9	9	9	9
10.....	5:00 P.M.	10	10	10	10
11.....	9:00 A.M.	11½	12	11	12
12.....	12:00 M.	12¼	12+	12	13
13.....	9:00 A.M.	12½	13	12	13
14.....	1:00 A.M.	13¼	13+	13	14

WATER METABOLISM

The water metabolism of the crayfish involves the uptake of water and ions via the gills and integument, and the excretion of a hypotonic urine by the green glands to maintain a blood which is hypertonic to the fresh-water environment. During the molting process of most crustaceans, including the crayfish, there is an increased uptake of water preceding molt. Experiments were performed to study the role of the sinus glands in some of these changes in water metabolism.

In one group of experiments, daily weight determinations for periods of 3–12 days were made on normal crayfish, animals with sinus glands removed, crayfish with eyestalks removed, and animals with eyestalks removed but with sinus-gland implants. The results of

TABLE 8

ANALYSIS OF SOLUBLE MATERIALS (CALCIUM COMPOUNDS) IN THE CARAPACE

Type of Carapace	No. of Cases	Av. Length (Mm.)	Av. Weight (Mg.)	Ratio: $\frac{\text{Weight}}{\text{Length}}$	Percentage Soluble Material and Standard Deviation
Intermolt	5	28.0	104.1	3.8	72.0±0.5
Just before molt	5	26.8	78.2	2.9	76.9±1.0
Normal, molted	9	28.0	78.5	2.7	78.5±1.6
Eyestalkless, molted	5	25.7	58.9	2.3	79.5±0.5

one such experiment (Table 9) revealed that eyestalkless crayfish increased in weight an average of 3.72 per cent of the body-weight. Sinus-gland ablation resulted in an increase of 1.48 per cent, whereas the weight of eyestalkless crayfish with sinus-gland implants was not

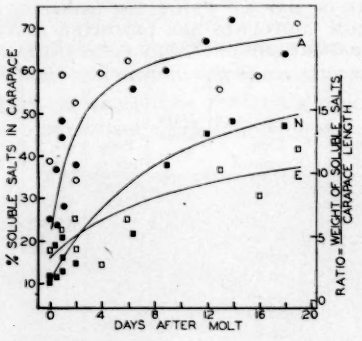

FIG. 5.—The rate of deposition of soluble compounds (mainly calcium) in exoskeleton following molt. Curve *A*, the change in percentage of soluble salts in carapace of normal (●) and eyestalkless (○) crayfish; curve *N*, the change in ratio of weight of soluble salts / carapace length in normal (■) crayfish; curve *E*, the same for eyestalkless (□) animals.

altered and the weight of normal animals decreased 1.14 per cent. Other experiments of this type resulted in weight increases of eyestalkless crayfish ranging from 1.26 to 6.83 per cent and decreases in weight of normal animals varying from 0.08 to 0.62 per cent.

In another experiment several crayfish were weighed, both eyestalks were removed from half of the crayfish, and a few animals of each group were sacrificed on the third and seventh postoperative days to determine total weight and water content. As indicated in Table 10, the eyestalkless crayfish increased in body weight up to 3.84 per cent, but the normal animals showed no appreciable weight change. The eyestalkless crayfish contained a higher percentage of water content than did normal ones, proving that the increases in body-weight were due to increases in water content. In general, experiments performed in the spring gave larger percentage increases in weight of eyestalkless crayfish than were noted in the fall.

Several eyestalkless crayfish and eyestalkless ones with sinus-gland implants were followed for a longer period of time, in order to study the changes in weight associated with molt produced by eyestalk ablation. The initial weight was determined for each animal, both eyestalks were excised, and weights were measured every 2 or 3 days for a period of as long as 3 weeks. The results of three individual cases are listed in Table 11. By the eleventh day there were marked percentage increases in body-weight of all the animals. The three animals listed were weighed when they were just beginning to molt. All the animals died during the molting process or shortly thereafter. Animal 13 died after completion of molting, and the weight of the freshly molted crayfish immediately after death showed

TABLE 9

WEIGHT CHANGES IN CRAYFISH

	Normal	Eyestalkless with Sinus-Gland Implants	Sinus Glands Removed	Eyestalks Removed
No. of animals	7	10	6	10
Av. initial weight (gm.)	10.49	9.89	8.92	9.68
Av. weight change (gm.)	− 0.12 ± .06	0.00 ± .06	+0.12 ± .05	+ 0.36 ± .07
Av. percentage change	− 1.14	0.00	+1.48	+ 3.72

TABLE 10

CHANGES IN BODY-WEIGHT AND WATER CONTENT

Experimental Condition	No. of Animals	Percentage Change in Body-Weight	Av. Percentage Water Content
A. *3 days postoperative:*			
Normal	8	−0.14±0.12	76.40±0.99
Eyestalkless	5	+1.86±0.64	76.94±2.96
B. *7 days postoperative:*			
Normal	7	−0.04±0.11	76.21±1.39
Eyestalkless	7	+3.84±0.61	80.36±1.59

an increase of 4.36 per cent over the premolt weight. The body-weight, including the exuvium at the time of molt, demonstrated an increase of 52.18 per cent over the original weight. In all three of these cases, the percentage of water content (80–86 per cent) was higher than in normal hard-shell crayfish (76 per cent) (Table 10).

Increases in body-weight of eight individual crayfishes following eyestalk removal are shown in curve *B*, Figure 6; and the increases in body-weight of a single eyestalkless crayfish with two sinus-gland implants are shown in curve *A*. There was an initial slow percentage increase in weight for about a week, followed by a rapid increase during the remaining days before molt. The curves followed rather closely the changes in gastrolith size and rate of oxygen consumption preceding molt in eyestalkless and normal crayfish. The sinus-gland implants retarded the rate of increase in weight but did not prevent it. These experiments were done in the spring of the year. This observation is interesting, since it was noted that sinus-gland implants did not completely suppress gastrolith formation in eyestalkless animals near the time of the spring molts.

GROWTH

One of the results of molting is an increase in the size of the crayfish. Increases in weight preceding and following molt have already been mentioned. Another measure of growth would be increases in carapace or total body-length. Complete records of carapace lengths were made before and after molt,

TABLE 11

CHANGES IN WATER CONTENT IN EYESTALKLESS CRAYFISH APPROACHING A MOLT

Animal No.	Days after Eyestalks Removed	Percentage Increase in Body-Weight	Percentage Water Content
29 (2 sinus-gland implants)	11	2.95	.....
	13	5.11	.....
	15	7.20	.....
	17	12.10	80.40
30	11	4.15	.....
	13	23.47	82.50
13	11	10.47	.....
	13	52.18	85.90
	..	4.36*	79.60

* After molt was completed.

which made it possible to analyze hormonal influences on growth. Several eyestalkless crayfish, which molted during the winter and lived long enough for the exoskeleton to harden, were measured for growth increment and compared with an equal number of normal crayfish from a regular spring molt. The results, as indicated in Table 12, revealed that a much greater increase in carapace length (5.6 per cent) occurred in eyestalkless cray-

fish following molt than occurred in normal animals (1.8 per cent) or in eyestalkless crayfish which received sinus-gland implants (1.55 per cent).

OXYGEN CONSUMPTION

Over one hundred individual experiments were performed to analyze any possible influence of the sinus glands upon metabolism as shown by changes in oxygen consumption. The results obtained in 1939 were generally the reverse of those obtained in 1940, 1941, and 1942 and reported in this paper. The difference was apparently one of refinement in techniques. It was noted in the early experiments that the rate of oxygen consumption gradually declined for the first 3 days before leveling off and that the first of a day's determinations was usually higher than subsequent ones. Therefore, in later experiments, the animals were placed in flasks in the temperature bath for 3 days prior to an experiment, and a mock determination was performed at the onset of each day's tests. Crayfish of both sexes, of carapace lengths ranging from 21.5 to 31 mm., and of body-weights varying from 2.2 to 8.0 gm., were used in these experiments.

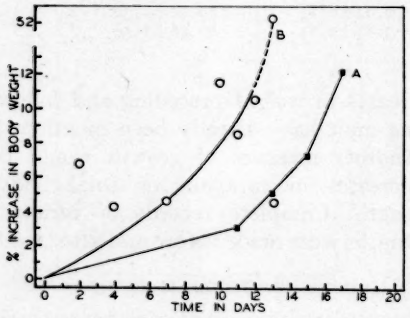

FIG. 6.—Increase in water content, as measured by changes in body-weight; curve *A*, a single eyestalkless crayfish (No. 29) with two sinus-gland implants; curve *B*, eight individual eyestalkless crayfish.

VARIATIONS DURING THE MOLT CYCLE

In the course of respiration experiments performed during the spring, eight crayfish (seven normal and one eyestalkless) molted. In these cases daily observations had been made, which included all or parts of the periods preceding and following ecdysis. In all experiments the animals were maintained in constant-temperature baths between tests and were not fed during the experiments, in order to eliminate variations due to temperature and food differences. A composite graph of the variations in oxygen consumption of four normal and one eyestalkless crayfish during the molt cycle is presented in Figure 7. The results demonstrated that the rate of oxygen consumption increased rapidly for a week preceding molt, reached a peak at about the time of molt, and decreased sharply for a week following molt, when a new basal rate was attained.

Animal 1 was observed for 7 days prior to molt and for 30 days after molt. The rate of oxygen consumption increased 49

TABLE 12

GROWTH MEASUREMENTS

Experimental Condition	No. of Animals	Av. Length of Carapace (Mm.)	Percentage Increase in Carapace Length
Normal (spring molt)	13	25.3±0.6	1.80±0.17
Eyestalkless (winter)	13	23.7±0.3	5.61±0.77
Eyestalkless, sinus-gland implants (winter)	2	23.5	1.55

per cent in a week to the level at the time of molt and then after molting decreased to 75 per cent below the molting level. Since the new basal rate after molt was much lower than the initial rate, 7 days before ecdysis, it would seem that the rate of oxygen consumption began increasing more than a week before molt. There was no essential difference in the changes in oxygen consumption preceding molt in a normal (No. 2) and an eyestalkless (No. 150) crayfish, which happened to weigh 4.5 gm. each. Both died at the time of molt or shortly thereafter. Animal 103, which was observed immediately after ecdysis and for several days thereafter, showed a decline in the rate of oxygen consumption to a new basal level.

Some evidence suggested that the rate of oxygen consumption might be less at the time of shedding the exuvium than just before or after molting. Animal 1 molted in the respirometer. Two hours before molt the rate of oxygen consumption was lower than on the previous day; during the molting process the rate was still lower, but 3 hours afterward the rate had returned to the previous high level. The oxygen consumption was determined in Animal 154 immediately after molting and was found to be lower than on the preceding and following days. It is possible that organic matter released during the molting process would interfere with oxygen determination by the Winkler method.

EXTIRPATION EXPERIMENTS

In these experiments the rate of oxygen consumption was determined on 1–4 successive days in order to obtain a basal preoperative rate, and then the changes in oxygen consumption were determined for 2–4 days after operation. This procedure was varied by making oxygen-consumption measurements immediately after cauterization of the eye stumps of eyestalkless animals in this particular experiment. The results, as condensed in Table 13, list the average rates of oxygen consumption with a statistical evaluation of the average percentage change in each experimental group.

Bilateral eyestalk extirpation of twenty-six animals resulted in an average increase of 59.2 per cent in the rate of oxygen consumption at the end of 2–4 days,

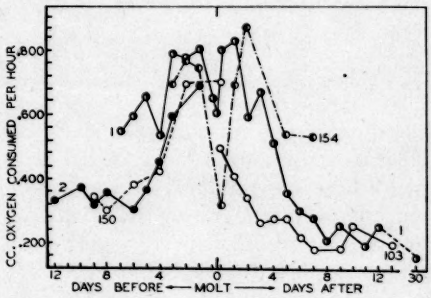

FIG. 7.—Variations in the rate of oxygen consumption during the molt cycle of the crayfish. All observations were made during spring molt of normal animals except No. *150*, which had both eyestalks removed.

whereas the excision of a single eyestalk produced a decrease of 11.9 per cent. Animals that had had no operation had an average increase of 0.9 per cent and animals with both antennae removed increased only 1.0 per cent in the rate of oxygen consumption. The results of eyestalk removal were essentially the same, regardless of whether the hemorrhage was controlled by cautery or by ligation at the base of the stalks. However, it was noted that cautery of the eyestumps of eyestalkless crayfish produced an average increase of oxygen consumption of 56.8 per cent, which had returned to basal levels when tested on the following day.

The effects of bilateral eyestalk re-

TABLE 13*

OXYGEN-CONSUMPTION DETERMINATIONS

Operation	No. of Animals	Av. Pre-operative Oxygen Consumption (Cc./Hr.)	Av. Post-operative Oxygen Consumption (Cc./Hr.)	Av. Percentage Change and Standard Deviation	Standard Error
Bilateral eyestalk extirpation	26	0.310	0.482	+59.2±18.9	±3.7
No operation	10	0.230	0.232	+ 0.9± 5.6	±1.8
Extirpation of one eyestalk	6	0.354	0.300	−11.9±18.0	±7.4
Excision of both antennae	9	0.410	0.378	+ 1.0±17.4	±5.8
Cautery of eye stumps of eyestalkless animals	4	0.433	0.677	+56.8± 9.3	±4.7
Bilateral sinus-gland removal	3	0.294	0.332	+13.0± 3.6	±2.4
Mock sinus-gland removal	1	0.226	0.166	−26.5	.....

* The few apparent discrepancies between the average oxygen-consumption values and the average percentage change in Tables 13 and 14 are artifacts due to the method of calculation. The statistical analysis was based on individual percentage changes, and, when there was a wide range in the oxygen-consumption values of individual crayfish in a certain group, the difference between the average preoperative and average postoperative consumption would not always agree with the average percentage change.

moval on oxygen consumption are better illustrated in Figure 8. Curve *E* indicates the average increase in oxygen consumption following bilateral eyestalk extirpation of six crayfish. Curve *S* shows the lack of significant change in oxygen consumption following removal of a single eyestalk in the individual crayfish and the marked increase after removal of the other eyestalk. Curve *N* reveals the average daily variations in oxygen consumption of six normal crayfish.

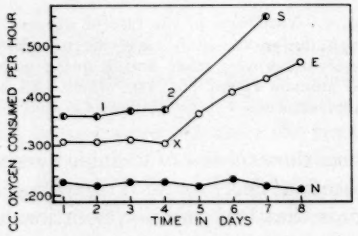

FIG. 8.—Changes in rate of oxygen consumption produced by bilateral eyestalk removal. Curve *N*, average of six normal crayfish; curve *E*, average of six animals with bilateral eyestalk ablation performed at *x*; curve *S*, one crayfish with one eyestalk removed at *1* and the other one at *2*.

A few tests were performed to determine whether the sinus glands were the source of this influence of eyestalk removal on metabolism. Bilateral sinus-gland ablation in three crayfish produced an average increase of 13 per cent in the rate of oxygen consumption. But mock sinus-gland excision in one animal resulted in a 26.5 per cent decrease, possibly due to stimulation which caused release of a sinus-gland hormone.

REPLACEMENT EXPERIMENTS

In these experiments two successive hourly determinations were averaged as the initial oxygen consumption and then one determination was performed after the injection of a tissue extract. The results are condensed in Table 14.

The injection of 0.05 cc. of a sinus-gland extract (10 glands/1 cc. saline) into each of fifteen eyestalkless crayfish produced an average decrease in oxygen consumption of 16.2 per cent. The injection of a saline solution or a muscle-tissue extract produced increases in oxygen consumption of 2.7 and 4.3 per cent, respectively.

The action of central-nervous-system tissues on oxygen consumption was investigated in a few tests. The injection of 0.05 cc. of an extract of supra-esophageal ganglia (1 "brain"/0.5 cc. saline) into

TABLE 14

OXYGEN-CONSUMPTION DETERMINATIONS

Injection Experiment	No. of Eyestalk-less Animals	Av. Initial Oxygen Consumption (Cc./Hr.)	Av. Consumption after Injection (Cc./Hr.)	Av. Percentage Change and Standard Deviation	Standard Error
Sinus-gland extract	15	0.473	0.396	−16.2±12.5	±3.2
Saline solution	10	0.295	0.301	+ 2.7± 5.2	±1.6
Muscle-tissue extract	5	0.331	0.346	+ 4.3±10.2	±4.6
"Brain"-tissue extract	5	0.352	0.434	+22.8±16.7	±7.5
Nerve-cord extract	8	0.352	0.455	+29.1±23.2	±8.2

eyestalkless crayfish produced an increase in oxygen consumption of 22.8 per cent. The injection of 0.05 cc. of an extract of thoracic nerve cord and ganglia (1 cord/0.5 cc. saline) resulted in an increase of 29.1 per cent. It was noted also that cautery of the eye stumps of eyestalkless crayfish, causing injury or stimulation of the "brain," produced an increase of 56.8 per cent in the rate of oxygen consumption, which was only transitory.

VIABILITY

One experiment was performed to check the viability-effect of the sinus glands. The survival time was determined in a group of eyestalkless crayfish and in a group of eyestalkless crayfish which had received sinus-gland implants. As revealed by Table 15, the average survival time after removal of both eyestalks was 16.91 ± 1.37 days. It was noted that many of the animals died when about to molt or in the molting process. However, whenever an animal molted successfully, it usually continued to live for several days. Nevertheless, in all cases, the eyestalkless crayfish soon died. No special attempts were made to sustain life, such as offering frequent feedings or maintaining water environment below room temperatures. The eyestalkless crayfish, which received a sinus-gland implant every 3 or 4 days for three to six implants, survived an average of 38.50 ± 5.84 days. Furthermore, the average survival time after the last implant in these animals was 24.75 ± 2.61 days, which was also greater than the average survival of the eyestalkless crayfish. This suggests that the sinus-gland implants were viable for at least a week. Viability in normal animals under similar conditions was not accurately determined, although one group was observed for 91 days without any deaths. During the winter an occasional normal crayfish died, especially when the water in the supply tank became too warm, stagnant, or overcrowded. Furthermore, several normal crayfish have been observed to die when about to molt or in the molting process during the regular spring and summer molting periods.

TABLE 15

SURVIVAL TIME IN EYESTALKLESS CRAYFISH

Experimental Condition	No. of Animals Observed	Av. Survival Time (Days)
Eyestalkless	58	16.91±1.37
Eyestalkless, with sinus-gland implants	10	38.50±5.84
After last implant	8	24.75±2.61

DISCUSSION

It is important to consider the normal molting process and life-cycle in studying

the effect of experimental factors on molting. In this investigation most of the experiments were performed during the winter intermolt period, in which molting does not normally occur, thus providing an excellent time to study hormonal influences on molting. Bilateral eyestalk excision instigated the formation of gastroliths and molting in a manner similar to that observed in the normal molting process. In addition, the removal of both sinus glands, with the rest of the eyestalk tissue left intact, initiated the formation of gastroliths. The implantation of sinus glands into the abdominal tissues of eyestalkless crayfish prevented the formation of gastroliths. These observations definitely establish the molt-inhibiting influence of the sinus glands.

Evidence was presented which suggested a diurnal rhythm in the deposition of layers during gastrolith formation after eyestalk ablation. Persistent diurnal rhythms have been reported in retinal-pigment migration (Welsh, 1941 and earlier; and others), in general motor activity (Kalmus, 1938*b;* Roberts, 1941; and Schallek, 1942), and in integumentary pigment migration or color changes (Kalmus, 1938*a;* and as reviewed by Welsh, 1938). However, in the case of motor activity and integumentary pigment migration, the diurnal rhythm generally disappeared after eyestalk removal. Therefore, the apparent diurnal rhythm in gastrolith formation of eyestalkless crayfish must depend on some nervous or hormonal influence other than the eyestalks or sinus glands. The observation that the injection of central-nervous-tissue extracts seemed to stimulate gastrolith formation was suggestive.

Changes in the composition of the exoskeleton occur with molting. No essential difference in the percentage of soluble salts (chiefly calcium) was noted in the molted carapaces of normal or eyestalkless crayfish. Kleinholz and Bourquin (1941*b*) made similar observations on *Palaemonetes;* but Koller (1930) and Plankemann (1935) found smaller amounts in the molted carapaces of eyestalkless crustaceans than in normal ones. It is reasonable and significant that the removal of the eyestalks (sinus glands) produced the same changes in the exoskeleton as occurred in the normal molting process. Both soluble compounds (calcium) and organic matter (chitin) are removed from the exoskeleton before molt. Thus, preceding molt in the crayfish, there is a resorption of calcium and chitin from the exoskeleton, an increase in the blood calcium (Numanoi, 1939), and deposition of calcium in gastroliths; after molt the gastroliths are dissolved, calcium is acquired from the environment (Maluf, 1940), the blood calcium is again elevated, and calcium and chitin are deposited in the exoskeleton. Apparently, the sinus glands are essential to complete the process of deposition of materials in the exoskeleton after molt.

Definite changes in water metabolism accompany the molting process and eyestalk removal. Bilateral eyestalk extirpation produced increases of weight up to 3.84 per cent in a few days, owing to increased water content; and sinus-gland removal produced similar changes. Sinus-gland implants prevented these increases in body-weight. Eyestalkless crayfish increased in weight a greater amount with time until, as in the case of one crayfish, a 52.2 per cent increase in weight was recorded at the time of molt. This value compares closely with the results of Maluf (1940). He happened to have recorded weights of one normal crayfish that molted and found that the weight of the body plus exuvium showed an increase of 51.5 per cent over the original body-weight. These increases in water content

could be due to increased uptake or decreased output of water or both; further experimentation is needed to elucidate these possibilities and the mechanisms involved. The early increases in water content following eyestalk extirpation seem slight, but they are significant in view of the constant volume maintained by the exoskeleton. As molt approaches, the increases in water content become marked and are indicated by bulging of the membranes between certain segments of the body and joints of appendages. It is entirely possible that changes in hydrostatic pressure play an important role in molting and initiating the shedding of the exuvium.

Striking variations in oxygen consumption occurred during the molt cycle, with an increase for several days preceding molt, followed by a decrease after molt. Bilateral eyestalk or sinus-gland ablation produced increases in oxygen consumption; and injections of sinus-gland extracts resulted in decreases in the rate of oxygen consumption. However, Helff (1928) reported no changes in oxygen consumption during the shedding of the exuvium in the molting process, but his experiments lasted only a few hours, whereas molting lasts several days. Maluf (1937) suggested that nervous or secretory factors might explain sudden drops in oxygen consumption, with changes in oxygen tension, noted in his experiments. It is plausible that release of the sinus-gland hormone could explain his observations.

It is interesting to note that sinus-gland implants were unable to retard the changes occurring after eyestalk extirpation in the spring (animals approaching spring molt period) as completely as they did in the winter (an intermolt period). This was found to be true in the case of gastrolith formation and of increases in water content. This situation could be explained by changes in the amount or kind of secretion from the sinus glands as suggested by the secretory changes observed by Pyle (1943) and by the probability that the factors which initiate molt have already been set in operation.

There were striking parallels in the variations of certain changes associated with molting in the eyestalkless crayfish. The formation of gastroliths, increases in oxygen consumption, and increases in water content all commence at least 2 weeks before molt, change slowly at first, and then increase rapidly the last 7 days before molt occurs. In all the processes studied (gastrolith formation, exoskeletal changes, water metabolism, oxygen consumption, and molting) there was marked similarity in the changes observed. Bilateral eyestalk or sinus-gland excision resulted in gastrolith formation, removal of soluble salts from the exoskeleton, increase in water content, increase in oxygen consumption, and molting; and sinus-gland implants or extracts prevented or retarded these changes. It must be concluded that either (*a*) all these processes are individually influenced by one or more sinus-gland hormones, or (*b*) all are associated as a single functional system and that this system is controlled by a hormonal substance from the sinus glands. No evidence was presented here to show a separate control of these changes different from the control of molting. A sinus-gland hormone has a molt-inhibiting influence; when the sinus glands are not producing this hormone or are removed, the physiological changes associated with molting are set in motion, ultimately resulting in a molt.

The factors responsible for initiating molt have not been elucidated completely in this or in previous investigations. The possibility of nervous or secretory

factors in or near the central nervous system having a molt-accelerating action is suggested by the observation that central-nervous-tissue extracts stimulate gastrolith formation and increase the rate of oxygen consumption in eyestalkless crayfish. In addition, the stimulation of the eye stumps of eyestalkless crayfish with the electric cautery raised the rate of oxygen consumption.

The greater increase in carapace length following molt in eyestalkless crayfish in the winter, as compared to normal animals in the spring molt, suggests a growth-retarding influence of the sinus glands. The increase of 5.6 per cent in carapace length of the eyestalkless crayfish after molt in the winter was not so great as the 10–30 per cent increases after the summer molt of normal animals reported by Van Deventer (1937) and Tack (1941) but was greater than the increases noted at the spring molt. It is entirely possible that food supply and other factors may influence these growth changes, because there is some starvation in the winter, in contrast to active feeding in the summer.

The viability-effect of the sinus glands was further emphasized. Eyestalk removal resulted in early death in every instance, and sinus-gland implants prolonged the life of eyestalkless crayfish. There was a close parallel between the time of molting (16.3 days) and time of death (16.9 days) after eyestalk extirpation. This observation is not surprising because it is known that molting is a difficult process and that many normal crayfish die during ecdysis—few animals live longer than 3 years (Van Deventer, 1937; Tack, 1941). The eyestalkless crayfish definitely cannot withstand adverse conditions so effectively as do normal ones. A theory which would explain why eyestalk (sinus-gland) removal results in the early death of crayfish would be to assume that disturbances of the water and oxidative metabolism (and probably other aspects of metabolism) in eyestalkless crayfish exert an important influence in their early death. The fatalities following eyestalk extirpation may be divided into four groups: (1) those that die in the first few days, probably because of operative injury; (2) those that die after several days, probably owing to an upset in metabolism; (3) those that die during the molting process because of mechanical and other factors; and (4) those that die after molting, probably because of an upset in metabolism. It seems possible that eyestalkless crayfish could be maintained for very long periods of time by keeping them in fresh running water, with a low temperature, adequate food supply, and frequent sinus-gland implants.

SUMMARY

1. The physiology of molting, the various changes associated with molting, and the life-cycle of the crayfish are reviewed.

2. A technique for the implantation of sinus glands is described.

3. The sinus glands have a growth-retarding effect, as shown by the greater increase in carapace length after molt in the eyestalkless crayfish, *C. immunis*, than in normal animals or eyestalkless ones with sinus-gland implants.

4. The viability-effect of the sinus glands is verified, and a theory to explain the early death of eyestalkless animals is presented.

5. The molt-inhibiting influence of the sinus glands is confirmed, and the influence of the sinus glands on changes associated with molting is elucidated.

6. Bilateral eyestalk or sinus-gland extirpation results in gastrolith formation and molting, and sinus-gland implants in eyestalkless crayfish prevent or retard the formation of gastroliths.

Removal of a single eyestalk fails to induce gastrolith formation.

7. There is a diurnal rhythm in the deposition of layers of calcium compounds in the formation of gastroliths in eyestalkless crayfish.

8. The molted carapaces of normal and eyestalkless crayfish contain essentially the same percentage of soluble compounds (chiefly calcium). Eyestalk extirpation results in removal of both calcium and chitin from the exoskeleton, which normally occurs before molt. The deposition of soluble salts (calcium) in the exoskeleton after molt is retarded in eyestalkless crayfish, suggesting that the sinus glands are essential for normal calcium deposition.

9. Bilateral eyestalk excision produces an increase in body-weight due to increased water content, and sinus-gland implants inhibit the ncrease in water content of eyestalkless animals.

10. There are variations in oxygen consumption during the molt cycle, with an increase preceding, and a decrease following, molt. Bilateral eyestalk or sinus-gland removal produces an increase in the rate of oxygen consumption. Injections of sinus-gland extracts decrease the rate of oxygen consumption, whereas saline solution or muscle-tissue extracts do not.

11. Central-nervous-tissue extracts ("brain" or thoracic ganglia) apparently stimulate gastrolith formation and produce an elevation in the rate of oxygen consumption, which suggests that the central nervous organs may be the source of a molt-accelerating influence.

12. Sinus-gland implants are ineffective in completely preventing gastrolith formation or in inhibiting the increase in water content after eyestalk ablation in the spring.

13. There are parallel changes in gastrolith formation, increase of water content, and increase in oxygen consumption before molt or following eyestalk extirpation. All these changes commence about 16 days prior to molt, proceed slowly for approximately 9 days, and then accelerate rapidly for the last week before molt occurs.

14. Molting and all these associated changes (gastrolith formation, exoskeletal changes, increase in water content, variations in oxygen consumption) seem to be related in a single functional system. A separate control of all these changes is not indicated. Thus these results suggest that a single hormonal substance from the sinus glands has an inhibiting influence on molting and the associated changes studied.

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NEW BOOKS IN PHYSIOLOGICAL ZOÖLOGY

[This section comprises reviews of selected new books containing new and significant material in the field of physiological zoölogy. Books submitted for review should be sent directly to the review editor: Frank A. Brown, Jr., Department of Zoölogy, Northwestern University, Evanston, Illinois.]

THE CHEMICAL SENSES,[1] BY R. W. MONCRIEFF

Reviewed by THEODORE LOUIS JAHN
State University of Iowa

THIS book, as the jacket blurb states, is unique. It is the only book in which an author has brought together most of our knowledge of the chemical senses (taste, smell, and general or common chemical sense). This is arranged in such a manner that the framework of that body of knowledge, together with its vacancies, is displayed to good advantage. If one wishes to learn about the chemical senses, this book is obviously the place to start. In many respects it is complementary to the old one by G. H. Parker (*Smell, Taste, and Allied Senses in Vertebrates* [1922]) in that Moncrieff emphasizes chemistry rather than physiology.

The book begins with a discussion of the role of the chemical senses in life and passes on to the structure and function of sense organs; then to each of the chemical senses of man and lower animals; then to a detailed classification of odors and the relation of chemical structure and physical properties to odor; then to a similar treatment of tastes; and then to a treatment of perfumes, essences, and flavor of food; and finally ends with a glossary.

The quality of treatment of the subject matter is highly variable, and at times the discussions seem to be uncritical. The sections on morphology and physiology were obviously not written by a professional in either field, and treatment of the subject matter is naïve in many instances. For example, it is stated that the "cerebellum . . . . controls activity and muscular movement"; and in this day of electronics only a capillary electrometer (coupled, however, to an amplifier) is described as the instrument for recording nerve impulses.

The chemical sections are excellent and contain the only extant review of the subject matter. The discussion of the relationship between chemical structure and odor and taste is inclusive, detailed, and well written. The author has attempted to formulate a set of principles which relate sensation to structure, and the enormity of the task (or at least of our present state of confusion about the matter) is obvious in that there are sixty-two principles enumerated for odor and fifty-eight for taste. Furthermore, there is a presentation of twenty-two theories of the perception of odors. These sections of the book are invaluable as a key to the literature and as a résumé of the known facts and principles.

However, the glossary is really a gold mine of incorrect and incomplete or misleading zoölogical definitions. Many of the incomplete ones are correct in only

[1] New York: John Wiley & Sons, 1946. Pp. 432. $4.50.

such a limited sense that they give an entirely erroneous idea of the subject. These will irritate any sensitive zoölogist and will attract the attention of even a casual reader, who has had some zoölogical training.

For example, "axone" and "axis cylinder" are both defined simply as "nerve fibre." "Dendrites" are "branching processes of a nerve." "Ganglia" are "swellings on nerves just before they enter the central nervous system." "Notochord" is the "precursor of the spinal cord." "Plasma" is "the living matter of a cell" as well as the liquid part of blood. "Protoplasm" is "the protein of which living matter is composed. Constitution not yet determined." The "corpus callosum" is "white matter of the brain." "Tissue" is "the substance of which organs are constituted." If these and other comparable definitions had been revised by almost any competent zoölogist, it would have added immensely to the value of the book. In most cases even consultation of a good dictionary would have been sufficient.

As a whole, the book is of good quality, and the amateurish approach to physiology does not prevent it from being a distinct and worth-while contribution to the subject of chemical senses. There are excellent subject and author indexes, and the author index is also subdivided as to subject—a very useful and convenient arrangement.

ANIMAL CYTOLOGY AND EVOLUTION,[1] BY M. J. D. WHITE

Reviewed by C. L. TURNER
Northwestern University

THIS book is a compact volume on the subject of the evolution of nuclear structures and the relationship of nuclear components to the process of evolution in animals. The author points out in his Preface that the subject of cytology has advanced so much and has become so divided into separate compartments since Wilson wrote his monumental book on *The Cell in Development and Heredity* that it would not be feasible now to include in one volume a general treatise on the subject of cytology. By excluding considerations of cell physiology, of cell phenomena other than those of the nucleus, of plant cytology, and the cytology of the Protozoa and by confining the subject to the Metazoan nucleus, it has been possible to include in one volume a thoroughly documented analysis of the literature on this subject.

Comparative structure and behavior of nuclear constituents are stressed in a critical review of the literature, but the twofold objective is continually held before the reader of, first, tracing the steps through which more complicated types have arisen from the simpler ones and, second, of noting the effects of the chromosomal evolution upon the course of evolution in the animals themselves. Most of the literature reviewed has been that of the last twenty years, but the author has not hesitated to go back as far as 1881 for certain key points of departure. The theoretical material has been renovated by setting aside without delay some of the obsolete and less likely postulates, but incisive reasons are given for abandoning such theories.

The Introduction is devoted in part to a clarification of the concept of specia-

[1] Cambridge, England: At the University Press; New York: Macmillan Co., 1945. Pp. viii+375. $7.50.

tion. Species are regarded as real and natural biological entities which have arisen by mutation or by chromosomal rearrangements and have become fixed by physiological, geographical, or ecological isolation.

In the main body of the text a succinct account of the structure and of structural changes in mitotic chromosomes is followed by a discussion of recent work on the salivary chromosomes of the Diptera. An explanation follows of the mechanism by which chromosomal rearrangements take place. In a chapter on meiosis particular attention is given to the function of chiasmata and to the establishment of new gene sequences by crossing-over in chromosomes which have undergone structural rearrangements. Another section treats the subject of chromosomal evolution in wild populations and in the genus *Drosophila*. "Standard" arrangements and rearrangements in chromosomal structure are considered with reference, first, to their present geographical distribution in wild populations and, second, to the steps through which derived types arose and are related to viability and the establishment of new species. Changes in chromosome form and number are assumed to have more significance in animal evolution than has been believed hitherto, because of the fact that gene sequences and position effects are altered when chromosomal changes occur. Changes in the standard process of meiosis are assumed also to be important for the reason that most anomalous conditions have resulted in the suppression of chiasma formation and crossing-over, with the consequent suppression of gene recombinations. The complicated subject of sex determination is fully treated from the viewpoint of the probable origins of the more complex types, but the author concludes that "the evolutionary significance of multiple sex chromosome systems is not clear." In one of the closing portions of the book the evolutionary consequences of the origins of different types of parthenogenesis are considered theoretically. The author returns, in conclusion, to the thesis that the origin of different genetic systems is the primary factor in the process of evolution and that extrinsic factors are secondary in the production of the end-products of speciation.

White's book is something of an English companion piece to Dobzhansky's *Genetics and the Origin of Species*. Both are concerned with modernizing and refreshing evolutionary theory, and both stress the genetic approach. The advanced student of zoölogy or genetics will find both the books essential because of the logical analysis contained in each and because of the extensive sweep of literature that is covered. The books will be useful also to teachers of biology who depend upon reviews and analyses to keep them abreast of new developments in fields in which they are not specialists.

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